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Myeloid Cell Actinomycin Binding in Human Myeloid Leukaemia

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Abstract Decreased actinomycin D binding was observed in the marrow cells of some patients with chronic myeloid leukaemia Blast uptake was still reduced in 2 cases of blast crisis and in acute myeloblastic leukaemia However the differentiating cells in blast crisis i e., those that had passed the blast stage, had exactly the same actinomycin D binding values as normal myeloid cells After 0.5 mg/kg methotrexate, increased actinomycin D binding on the part of the entire leukaemic population was observed in one case of acute myeloblastic leukaemia. These findings suggest differences in chromatin behaviour and genetic activity in myeloid cells in the different stages of myeloid leukaemia.

Key Words
Actinomycin binding
Blastic crisis
Genetic regulation
Methotrexate
Myeloid cells
Myeloid leukaemia

Several metabolic abnormalities in both chronic and acute myeloid leukaemia have been described in recent years In chronic forms, the existence of a proliferative disorder due to the loss of mechanisms regulating the homeostatic equilibrium of the stem cell population has been suggested [1] Many abnormalities in the proliferation-maturation compartment have also been reported [2–8]

Acute leukaemia, on the other hand, is essentially characterized by defective differentiation Its cell population is distinguished by abortive maturation whereby large, proliferating blasts give rise to small, mostly non proliferating blasts [9] The result is a kinetically heterogeneous population furthermore, some non proliferating blasts may be reactivated and re-enter the cycle [10] This pattern has recently been expressed in a three-compartment model [11]

These various abnormalities, if considered as intrinsic cell lesions, could be ascribed to transcription and/or translation defects. In this connection, the binding of actinomycin to chromatin, which is an index of the amount of free DNA, is closely related to genetic activity, or at any rate to the physicochemical characteristics of the DNA-protein complex [12–15]. The autoradiographic method can be usefully employed with such highly heterogeneous materials in order to obtain a valid comparison between normal and leukaemic cells [16].

The present study reports preliminary observations on differences in chromatin activity, expressed as actinomycin-binding values, in several cases of chronic myeloid leukaemia (CML) – two of them being in blast crisis – and acute myeloblastic leukaemia (AML). Comparison was made with corresponding normal bone marrow cells.

Materials and Methods

The method of BRACHET and FICQ [17] was used for the autoradiographic evaluation of ^{3}H actinomycin binding by the cells of normal bone marrow (5 cases), CML (4 cases), blast crisis in chronic myeloid leukaemia (BC, 2 cases) and AML (2 cases). All cases were in a relatively steady state and no cytostatic treatment had been previously administered in the CML and AML forms.

In 1 case of AML (No. 7), actinomycin binding was evaluated before, and 62 and 72 h after administering two doses of methotrexate, totalling 0.5 mg/kg.

Mean grain counts (MGC) were made on an average of 100 cells. An evaluation of actinomycin binding was also made for three equally spaced cell diameter classes (small, medium and large acute leukaemia blasts). In case 7, MGC were also determined for cells with 2n (blasts in G_0, G_1 and Z) and 2n-4n (blasts in S-G_2 phase) DNA contents, as evaluated by cytophotometry after Feulgen staining.

Results

A significant decrease in actinomycin D binding was observed in the majority of the CML myeloid cells (table I). Differences in behaviour between marrow and peripheral cells were also found, peripheral values were, in fact, sometimes equal to (cases 1 and 3) and sometimes lower than (cases 2 and 4) those of the corresponding marrow cells (table I).

A marked decrease, as compared to normal marrow blasts, was also noted in the 2 cases of BC (No. 5 and 6), where the blasts represented 60–70% of the total population (table I). In case 5, the more mature mye-

Table I Actinomycin binding in marrow and peripheral cells, in patients with CML, BC and AML

		Blasts MCG	Promyelocytes MGC	Myelocytes MGC	Metamyelocytes MGC	Granulocytes MGC
Normal marrow	(control)	100±10 3	85±17.0	59±10 7	48±13 0	47±10 7
Case 1 (CML)	marrow	41±6 7**	42±11 6**	27±5 8**	22±3 6**	22±4 1**
	peripheral	40±7.6**	40±9 8**	31±6 2**	25±7 6**	20±8 0**
Normal marrow	(control)	100±30 7	96±17 6	55±8 8	44±8 9	46±8 8
Case 2 (CML)	marrow	68±13.2*	73±13 1**	40±9 2**	30±8 8**	27±4 4**
	peripheral	54±17 6**	48±13.2**	32±8 8**	22±4 4**	21±8 3**
Normal marrow	(control)	100±23 8	105±31 8	69±18.5	52±11 5	52±15 9
Case 3 (CML)	marrow	74±6 2*	63±14 1**	43±9 7**	32±7 0**	31±3.5**
	peripheral	78±12.3*	74±11.5**	51±8 8**	40±9 7**	38±4 4**
Normal marrow	(control)	100±19 5	84±19 3	75±13 0	50±7 6	50±4 4
Case 4 (CML)	marrow	84±16 0	78±16 1	73±19 6	46±8 3	35±8 4**
	peripheral	54±16 4**	52±10 5**	32±6 3**	27±6 5**	27±5 8**
Normal marrow	(control)	100+19.5	87±19.3	54±13 0	44±7 6	40±4 6
Case 5 (BC)	marrow	70±23 8**	83±23 9	54±10 8	43±13 0	42±6 4
	peripheral	77±19 5**	81±21 7	54±18 2	48±13 1	48±9 5
Case 6 (BC)	marrow	73±24 0**	–	–	–	–
	peripheral	63±26 0**	–	–	–	–
Case 7 (AML)	marrow	38±7.2**	–	–	–	–
	peripheral	20±7 6**	–	–	–	–
Case 8 (AML)	marrow	42±13 4**	–	–	–	–
	peripheral	24±10 0**	–	–	–	–

Values are compared with those observed in normal marrow and expressed in arbitrary units (*MGC for* normal marrow blasts = 100) The asterisks indicate significant (*) and highly significant (**) differences in binding values, between myeloid leukaemia cells and the r corresponding normal cells

loid cells presented almost the same values as the corresponding normal cells In both cases, marrow and peripheral blood binding values were identical

In AML (cases 7 and 8), the binding values were much lower than those for normal bone marrow blasts (table I), the lowest values being found in the peripheral blood Significant differences were also observed between proliferating (large) and non proliferating (small and medium) blasts, with higher actinomycin binding values for the former (table II)

Treatment with methotrexate in AML (case 7) led to a fall in both

Table II Actinomycin binding in AML cells, subdivided in three equally spaced diameter classes

		Total population MGC	Large blasts MGC	Medium blasts MGC	Small blasts MGC
Case 7 (AML)	marrow	38±7 2	44±10 6	36±9 4	34±7 6
	peripheral	20±7 6	24±12 2	18±7 2	18±9 8
Case 8 (AML)	marrow	42±13 4	60±14 4	44±13 0	36±5 2
	peripheral	24±10 0	30±12.2	22±7 8	16±2 8

marrow and peripheral cellularity and to an increase in ^{3}H thymidine labelling from 3 to 10%. A marked increase in actinomycin D binding was noted in all three blast classes (table III) This increase was equally noticeable in the 2n (23%) and 2n–4n (45%) DNA cell classes

Discussion

Most CML cells showed reduced actinomycin binding by comparison with their corresponding normal cells, suggesting a decrease in genetic activity This finding agrees with earlier reports on the kinetic and biochemical abnormalities in such cells reduced proliferative activity [1–5], ineffective myelopoiesis [1], reduced nucleus-to-cytoplasm RNA transfer [4] and generation of less effective granulocytes, with defects both quantitative and qualitative, in alkaline phosphatase activity [6–8]

What further changes in cell function are responsible for the terminal BC? The Philadelphia anomaly is still present and it may be supposed that regulation of genetic activity deteriorates to the point where most cells become incapable of differentiation By contrast with the pre-crisis population, the small number of myeloid cells that pass the critical blast step and become capable of differentiation are more efficient in genetic activity their actinomycin-binding values increase to reach those of normal cells, while the granulocyte alkaline phosphatase activity usually shows a marked increase These findings may indicate a wide range of genetic activity in the Ph⁺ clones during the course of the disease It is also possible that the residual, differentiating cells, are cytogenetically normal,

Table III Changes in actinomycin binding, after methotrexate, in one case of AML

Case 7 (AML)	Total population marrow MGC	Total population peripheral MGC	Marrow large blasts MGC	Marrow medium blasts MGC	Marrow small blasts MGC	Peripheral large blasts MGC	Peripheral medium blasts MGC	Peripheral small blasts MGC
Before treatment	38 ± 7 2	20 ± 7 6	44 ± 20 6	36 ± 9 4	34 ± 7 6	24 ± 12 2	18 ± 7 2	18 ± 9 8
62 and 72 h after i v methotrexate (2 doses, totalling 0 5 mg/kg)	92 ± 12 2	64 ± 19 0	100 ± 21 8	92 ± 22.0	83 ± 18 4	88 ± 21 6	60 ± 21 4	50 ± 17 0

i e , Ph⁻ Subnormal binding values, together with a loss of differentiative activity, are noted for the blast population during such crises these cells will gradually lose the power to synthesize RNA, DNA and proteins, and will die as blasts [9]

Blast actinomycin binding is also highly reduced in the later stages of overall blast transformation and in primary acute leukaemia, with a significant and still greater decrease on the part of the non-proliferating compartment The fact that the values for the proliferating compartment are also below those found for normal marrow makes it clear that the decrease is not solely attributable to the presence of large numbers of non-proliferating cells It is more probably an expression of considerable physical and chemical chromatin changes, leading to altered genetic activity

In one case of AML, a marked increase in actinomycin-D-binding values for both large and small blasts followed administration of the phase-specific antiblastic drug, methotrexate As already shown [18], there was an appreciable increase in the number of cells in S phase This could be due to both blocking by the drug and re-entry of non-proliferating cells to the cycle [10–19] There is evidently a form of incompletely effective growth control in acute leukaemia populations, which is dependent on interactions between cells and their environment A cell not only contributes to its own environment, but can also display a selective response, e g , replication, to changes in this environment This is particularly evident in the case of the reduction of leukaemia populations after a cytostatic treatment In these cases, the sizes of both the proliferating and the non proliferating compartments are diminished and a certain number of cells in G_0, potentially capable of mitosis, are available for re-entry into the cycle This quota, in its passage from G_0 to G_1, expresses its particular phenotype as a highly undifferentiated population (i e , capable of nothing more than mitosis) and initiates a new 'chromosome cycle' [20, 21] Chromatin is decondensed or modified in some way with a consequent increase in genetic transcription, expressed by enhanced actinomycin binding Such an increase can also be shown for the small diameter blasts with a 2n content in DNA, since they are already activated in G_1 and thus involved in the cell cycle

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Acta Haemat 51 9-18 (1974)

Secondary Anemia (XV) and Reticuloendothelial Uptake in Cancer[1]

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Abstract An attempt was made to study the relationship between secondary anemia and the reticuloendothelial uptake in 18 patients with cancer The RE uptake of colloidal radiogold was significantly more rapid in the patients than in the controls, so was the plasma clearance of radioiron even though there was not any statistically significant anemia The gold and iron clearances were significantly correlated to each other, and to the erythrocyte sedimentation rate The results are considered to be compatible with the hypothesis that cancer anemia may be caused, e g., by RE uptake of some erythropoietic substances and of erythrocytes

Key Words
Anemia in cancer
Erythropoiesis
Iron metabolism
Radiogold
RES uptake

In previous studies, two different sets of metabolic disturbances in tumor patients were described, which could perhaps explain the secondary anemia in these patients One part of these earlier studies demonstrated that in patients with leukemia and cancer the plasma clearance of folic acid [9, 10, 27, 29] is accelerated, with a decreased serum concentration as a result Similar findings were made regarding iron [22, 25] and it was also shown that there are significant correlations between the metabolism of iron and that of folate [10] Previous studies have also shown that these metabolic changes are not specific for patients with cancer, similar findings being made in patients with tissue damage of an inflammatory [11, 37] or traumatic [21] nature However, the cause of these metabolic changes has not been identified

Another part of the previous studies [2, 7, 8, 15, 16, 23, 26, 31, 34-36] deals with the possible role of the reticuloendothelial (RE) system

[1] Supported by the Swedish and Stockholm Cancer Societies

[2] International Atomic Energy Agency Fellow from University of Bucharest, Romania.

in the host reaction to neoplasms. There are relatively many indications that an activation of the RES is seen in animals with experimental tumors [2, 26, 34] and also in patients with tumors [31, 36]. These studies have been reviewed [3, 26, 35].

So far, no simultaneous study is available of the hematological data and the data concerning the RE uptake in cancer patients. The present purpose was to study these relationships since they may be important for the understanding and control of the anemia and the nutritional disturbance in patients with cancer and inflammation.

Material and Methods

18 cancer patients and 8 controls were studied. The patients were aged between 40 and 80 years (mean age 61.5 years) and had different types and localizations of cancer and different general conditions (table I). Nine patients had metastases, 8 presented weight loss and 4 were examined shortly after operations. However, since neither for iron elimination nor for reticuloendothelial uptake any significant or even appreciable numerical difference could be found between the operated and the nonoperated patients, or between those with and without metastases, this group of cancer patients was treated as a single group. The results in this group were compared with those obtained in a group of 8 healthy volunteers with a mean age of 67.2 years.

The patients were subjected to a study of the RE uptake of a small dose of 0.05–0.5 mg colloidal ^{198}Au as previously described [38]. In an attempt to distinguish a rapid RE uptake caused by a high hepatic blood flow [24] from one caused by a high phagocytic uptake, the same patients got 12 μCi ^{131}I rose bengal (Amersham). The ^{59}Fe clearance was estimated by giving 4–5 μCi ^{59}Fe-citrate (Studsvik, Sweden), corresponding to 0.15–0.30 μg of iron. The folate clearance was estimated as described previously by giving folic acid (Folvite, Lederle) intravenously in a dose of 15 μg/kg body weight, and by assaying subsequent plasma samples as described [28].

In interpreting folic acid eliminations, both the level and the slope (FA_k) of the curve were considered. The level was given as the 15 min concentration (FA 15'). A further description of the measurements of radioactivity, calculations, etc. is given in a previous study [38].

The hemoglobin concentration (Hb), white blood cell and differential counts, erythrocyte sedimentation rates (ESR) and in some cases serum electrophoreses, cholesterols (chol.), irons (Fe) and transaminases (GOT and GTP) were performed by routine methods in the central clinical laboratory.

Results

Significantly increased values were found in the cancer patients in the plasma clearance rates of gold and iron (table II). The erythrocyte sedi-

Table I Description of patients with cancer

Case No	Sex	Age, years	Diagnosis	Microscopic examination	Weight loss	Known metastasis	Hb g/ 100 ml	ESR mm/ h	Operation *before study*	Treatment at time of study	Other observations
1	F	58	*Ca mam. dx.*	low differentiated adenocarcinoma	–	–	12.5	3	ablatio mam. *dx.* (1 week before)	–	8 years before ca mam sin operated
2	F	58	Ca mam sin	low differentiated adenocarcinoma	–	–	13.0	18	–	–	–
3	M	58	Ca ventriculi	low differentiated adenocarcinoma	marked (11 kg in 1 month)	liver and peritoneum	11.9	52	explorative laparotomy (1 week *before*)	–	–
4	M	60	Ca cardiae	–	–	–	12.6	73	–	–	–
5	M	59	Ca pancreatis	medium differentiated adenocarcinoma	marked (20 kg in 1 month)	–	14.2	–	*explorative* laparotomy (*1 month* before)	–	–
6	M	74	hypernephroma renis bilat	–	–	–	11.8	8	–	–	malignant ascites cells
7	M	52	Ca pelvis renis sin	highly differentiated adenocarcinoma	slight	–	11.8	6	nephrectomia *sin* (1 week before)	antibiotics	pneumonia at time of study (no fever)
8	M	79	Ca of urinary bladder	low differentiated	moderate	–	8.7	110	–	antibiotics	urinary infection, blood transfusions recently given

Table I (continued)

Case No	Sex	Age, years	Diagnosis	Microscopic examination	Weight loss	Known metastasis	Hb g/100 ml	ESR mm/h	Operation before study	Treatment at time of study	Other observations
9	M	78	Ca prostatae	medium differentiated adenocarcinoma	–	vertebrae, pelvis, liver, mediastinum	12 5	30	–	anabolic steroids (Stilbol 5 mg × 1) for 2 years	blood transfusions recently given
10	M	40	Ca testis dx	embryonal carcinoma and seminoma	–	–	10 4	34	retroperitoneal lymph node resect (11 days before)	–	–
11	M	63	Ca pulm	undifferentiated cell rich tumor	slight	lymph nodes, liver	13 0	46	–	–	4 months earlier anabolic steroids were given
12	M	54	Ca pulm bilat	adenocarcinoma	moderate	lymph nodes, miliary spread in lungs and pleura	12.7	48	–	–	bilat hydrothorax
13	M	52	Ca pulm	squamous epithelial type low diff	–	lymph nodes	13 0	60	–	–	–
14	F	66	Ca pulm	small cell type	moderate	hilus lymph nodes, kidney	13 0	92	–	–	–

Table I (continued)

Case No	Sex	Age, years	Diagnosis	*Microscopic examination*	Weight loss	Known metastasis	Hb g/100 ml	ESR mm/h	*Operation before study*	Treatment at time of study	Other observations
15	F	42	Ca pulm.	medium diff bronchiolar-alveolar cell-adenocarcinoma	slight	lymph nodes	13.0	51	–	–	5 months earlier partial tumor extirpation and cytostatic treatment
16	F	80	haemangiosarcoma hepatis	*uncertain results*	–	–	14.2	45	–	–	–
17	M	78	fibrosarcoma testis dx	–	–	local recurrence	13.7	59	*6 weeks before op of local recurrence*	–	first op Dec 1965 4 local recurrences op since
18	M	55	melanosarcoma regio dorsi	–	–	lymph nodes	14.7	3	4 weeks before resect of lymph node metastasis	–	primary tumor op 1 year before

Ca = Cancer, – = not known

Table II RE uptake, iron clearance, etc in the patients with cancer and in the controls

Parameter	No	Control group M±SE	No	Cancer group M±SE	Statistical significance
Age, years	8	67.2±3 4	18	61 5±3 9	–
Hb, g/100 ml	7	13 4±0 3	18	12 6±0 3	n s
ESR, mm/h	7	19 3±5 1	18	45 1±6 9	0 01<p<0 05
RE uptake of gold	8	0 16±0 014[2]	18	0 30±0 032	0 001<p<0 01
Iron clearance	8	0 008±0 0007	18	0 016±0 0015	p<0 001
Rose bengal clearance rate, first slope[1]	8	0 12±0 012	13	0 14±0 013	n s
Rose bengal, second slope	8	0 012±0 0009	13	0 018±0 001	0 01<p<0 05
Folate clearance, serum folate 15 min after inj	5	72 0±19 2	12	41 1±9 7	n s
Total serum proteins, g/100 ml	5	6 82±0 02	13	7 57±0 18	0 01<p<0 05
α_1 Globulin	5	0 33±0 02	7	0 46±0 04	0 01<p<0 05
α_2-Globulin	5	0 64±0 04	7	0 92±0 04	p<0 001
β-Globulin	5	0 54±0 01	7	0 93±0 14	0 01<p<0 05

[1] All clearances except folate are expressed as rate constants The gold and rose bengal clearance sometimes has two slopes, the corrected first slope is given here

[2] Corresponding to $T^1/_2$ 4 1±1 (S D) min

mentation rate and the serum concentrations of α_1-, α_2-, and β-globulins were also significantly increased (table II) Numerical, but statistically not quite significant decreases were found for the serum iron, albumin and folate concentration In addition to these differences some significant correlations were found The more rapid the colloidal gold elimination was, the more rapid was the iron elimination As in earlier studies, the iron elimination was also correlated to the erythrocyte sedimentation rate (fig 1), and the second slope of rose bengal (RB_{kII})

Discussion

Discussion of the method The RE activity is not well defined [6] To study it quantitatively the elimination rates from the blood of colloidal carbon [2, 19, 26, 34], gold [1, 8, 35], albumin aggregates [4, 7, 17, 36],

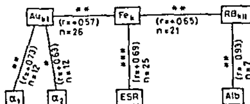


Fig 1 Correlation between RE uptake, iron clearance, etc in the controls and the patients with cancer * = 0.05>p>0.01, ** = 0.01>p>0.001, *** = 0.001<p, n = number of pairs r = correlation coefficients Signs indicate positive or negative correlations The figure shows that the more rapid the RE uptake of radiogold (Au_{kI}) is, the more rapid is also *the radioiron clearance rate* (Fe_k) and the higher are the α globulin concentrations (α_1 and α_2) The more rapid the iron clearance (Fe_k) is, the higher is also the erythrocyte sedimentation rate (ESR) the more rapid is the *late* clearance of rose bengal (RB_{kII} [40]) and the lower is the serum albumin concentration (Alb)

lipids [5, 20, 31] and labeled erythrocytes [12, 13, 32] have been used It is not known which aspects of the physiologic uptake by the RE cell these substances reflect. Colloidal gold was chosen here because it is of reasonably uniform particle size, disappears rapidly from the blood when given intravenously, and is almost entirely taken up by the RE system [35] However, when small doses of gold colloid are given, the disappearance might reflect liver circulation [30] rather than the phagocytic activity For this reason the term RE uptake is used

An attempt was made to distinguish increased hepatic circulation from an increased phagocytic activity Initially after injection, rose bengal, being specifically taken up by hepatic cells, reflects hepatic circulation, but no rapid clearance was found at this time Moreover, since an increased liver circulation in the present patients was not to be expected, an increased RE activity is the more likely explanation of the present results

Discussion of the results The previous results regarding RE uptake in patients with tumors are contradictory Several earlier studies in cancer patients did find signs of an increased RE uptake [31, 33, 36], while others have found a low RE uptake in cancer [7, 8] The different results probably depend upon the various methods used, the clinical condition of the patients, and the type and spread of the tumor, etc.

The coexistent increase of RE uptake and of the plasma elimination of iron in patients with neoplastic disease raises the question if iron and/or erythrocytes are taken up by RE cells This question has been discussed

earlier [21, 38, 39] In chronic inflammatory diseases iron is rapidly eliminated from the plasma and can be demonstrated histochemically in the RE cells in both bone marrow and liver. In all secondary anemias there is also a reduced red cell life span [14], sometimes correlated to the plasma iron clearance [21]. Thus it is reasonable to assume that the rapid elimination of iron is, like the high rate of clearance of colloidal gold, caused by reticuloendothelial uptake. It is of particular interest that the RE uptake was significantly increased in the present patient group, which did *not have any statistically significant anemia.*

The anemia secondary to tumors is caused not only by moderate hemolysis, but also by decreased red cell formation [14]. One possible cause for the decreased formation could be an increased RE uptake of erythropoietic factors like iron and folate [27]. However, this study does not constitute proof that the disappearance of iron and folate are actually caused by increased RE uptake. It is also possible that both phenomena may have common causes [18].

The late clearance of rose bengal, during the second part of curve, was rapid like in previous studies. The interpretation of this fact will be discussed separately [40].

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earlier [21, 38, 39] In chronic inflammatory diseases iron is rapidly eliminated from the plasma and can be demonstrated histochemically in the RE cells in both bone marrow and liver In all secondary anemias there is also a reduced red cell life span [14], sometimes correlated to the plasma iron clearance [21] Thus it is reasonable to assume that the rapid elimination of iron is, like the high rate of clearance of colloidal gold, caused by reticuloendothelial uptake It is of particular interest that the RE up take was significantly increased in the present patient group, which did not have any statistically significant anemia

The anemia secondary to tumors is caused not only by moderate hem olysis, but also by decreased red cell formation [14] One possible cause for the decreased formation could be an increased RE uptake of erythro poietic factors like iron and folate [27] However, this study does not constitute proof that the disappearance of iron and folate are actually caused by increased RE uptake It is also possible that both phenomena may have common causes [18]

The late clearance of rose bengal, during the second part of curve was rapid like in previous studies The interpretation of this fact will be discussed separately [40]

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Synthese von Hämoglobin, RNS und DNS bei hämolytischer Anämie, Thalassämie und akuter Blutungsanämie[1,2]

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Key Words
Cytophotometry
DNS synthesis
Hemoglobin formation
Hemolytic anemia
Nucleic acids
RNS synthesis
Thalassemia

Abstract In hemolytic anemia acute hemorrhagic anemia and β thalassemia, transformation of stem cells into morphologically identifiable red cell precursors already yields, in part, smaller erythroblasts during a brief G_1 phase The interphasic RNA influx into the cytoplasm is increased in hemolytic and hemorrhagic anemias and diminished in β thalassemia. In hemolytic anemia and acute hemorrhagic anemia, interphasic hemoglobin synthesis occurs in young erythroblasts as in normal erythropoiesis it is reduced to one half in thalassemia. In the cases examined the critical hemoglobin concentration' is lower in the G_2 phase erythroblasts than in normal erythropoiesis, and frequently reached in young erythroblasts. Thus the number of maturation stages is reduced

Aufgrund quantitativer UV-photometrischer Untersuchungen verschiedener Zellparameter konnten wir an der normalen Erythropoese folgende Befunde erheben Die Stammzellumwandlung in morphologisch erkennbare rote Vorstufen erfolgt in der G_1-Phase überwiegend in grosse Erythroblasten Die stärkste RNS-Synthese erfolgt in den jungen Erythroblasten, Hämoglobin- und RNS-Neubildung gehen auch noch in teilen und nicht mehr teilungsfähigen Erythroblasten weiter Eine kritische Hämoglobinkonzentration, die nicht überschritten wird, wird in der

[1] Mit Unterstützung der Deutschen Forschungsgemeinschaft.
[2] Herrn Prof Dr Dr h.c. H E. BOCK zum 70 Geburtstag gewidmet

G_2-Phase überwiegend in den kleineren Erythroblasten erreicht Die Befunde zeigen, dass zwar die Mehrzahl der Zellen eine gleiche Zahl von Generationszyklen bis zur Erythroblastenreifung durchlaufen dürften, dass aber auch in der normalen Erythropoese eine unterschiedliche Zahl von Generationszyklen anzunehmen ist [13] In der vorliegenden Arbeit soll geprüft werden, welche Veränderungen die genannten Parameter in der pathologischen Erythropoese erfahren

Methodik

Die Untersuchungen erfolgten an auf Quarzobjektträgern ausgestrichenen Knochenmarkpunktaten wie in der vorangegangenen Arbeit angegeben [13] Untersucht wurden jeweils 350 Erythroblasten Die Ergebnisse wurden mit den an der normalen Erythropoese erhobenen Befunden [13] verglichen Mit Hilfe von Computerprogrammen wurden jeweils die verschiedenen Zellparameter zueinander in Beziehung gesetzt und die Erythroblasten zum einen aufgrund des DNS(+RNS) Gehaltes des Zellkernes in Erythroblasten der G_0/G_1 Phase, der S- und G_2 Phase bzw in drei willkürlich herausgegriffene die Zellreifung repräsentierende Erythroblastengrossenklassen (bis 32 bis 60 und über 60 μm^2) eingeteilt

Krankengut

Die Befunde der untersuchten Patienten mit schwerer hämolytischer Anämie bei metastasierendem Magenkarzinom (Deu) mit Thalassaemia minor (Mo) sowie mit einer schweren Blutungsanämie vor Blutsubstitution (End) gehen aus Tabelle I hervor Bei dem Kranken mit hämolytischer Anämie war 3 Tage zuvor die letzte Bluttransfusion erfolgt bei dem Patienten mit Thalassamie waren Hamoglobin und Erythrozytenzahl über mehrere Wochen konstant gewesen Die Sternalpunktion bei dem Kranken mit Blutungsanämie erfolgte 2 Tage nach Einsetzen einer Ulkusblutung

Ergebnisse

Hämolytische Anämie Bei der Karyometrie fällt das Fehlen kleiner Normoblasten auf Die Kernflächenverteilung zeigt im Vergleich zur normalen Erythropoese [12] keine Maxima Die Kerngrösse nimmt wie in der normalen Erythropoese von der G_1- über die S- zur G_2-Phase zu Der Zellanteil mit erhöhtem DNS Gehalt ist grösser als in der normalen Erythropoese

Die RNS Synthese ist in den jüngsten (grössten) Erythroblasten während der Interphase gegenüber der normalen Erythropoese gesteigert (Abb 1, oben) Nicht messbare Mengen von Hämoglobin finden sich

Tabelle I Klinisch hämatologische Befunde bei Patienten mit hämolytischer Anämie (Deu) β-Thalassämie (Mo) und akuter Blutungsanämie (End)

	Name		
	Deu	Mo	End
Alter, Jahre	16	30	59
Blut			
Erythrozyten, Mill/mm³	2,16	5,21	1,79
Ery Volumen, µm³	94	68	109
Hb, g%	7 0	11,3	6 8
Hbe, pg	33,2	31 6	33 0
Hb F, %		2	
Hb A₂, %		5,4	
Retikulozyten ‰	162	17	43
Leukozyten/mm³	12600	5600	12000
Thrombozyten/mm³	133000	234000	410000
LDH, IE	1121	120	230
Bilirubin, mg%, gesamt	3,2	0 8	0,3
direkt	1,9		
Knochenmark			
Anteil roter zu weissen Zellen	300	54	45
Sideroblasten, %	10	42	30
PAS *in roten Vorstufen*, %	0	6	4

nur in wenigen grossen, zum Teil auch kleineren Erythroblasten Sie befinden sich aufgrund des Kern DNS-Gehaltes in der G_1-Phase Ihr Anteil ist in den jungen (grossen) Erythroblasten geringer als in der normalen Erythropoese (Abb 2, oben) Die interphasische Zunahme des Hamoglobingehaltes geht aus Abbildung 2 für die Gruppe der grossen (jungen) Erythroblasten hervor In der G_1-Phase wird eine bestimmte Hämoglobinkonzentration (Hamoglobingehalt/µm³) nicht überschritten und nicht nur in kleineren d h älteren Erythroblasten sondern auch in jungeren Zellen mit relativ grossem Zellkern erreicht (Abb 3) Diese «kritische» Hamoglobinkonzentration liegt niedriger als in der normalen Erythropoese *Eine weitere Hamoglobinzunahme erfolgt in den kleinsten* noch ausreifenden Erythroblasten

β-Thalassämie Die RNS-Zunahme im Zytoplasma ist während der Interphase in den jungsten (grössten) Erythroblasten am deutlichsten.

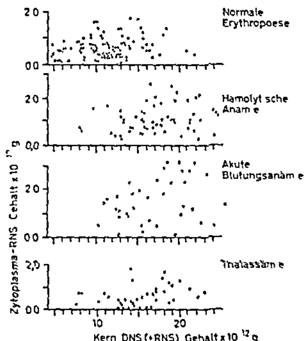


Abb 1 Zunahme des Zytoplasma RNS Gehaltes während der interphasischen Kern DNS-Synthese in der Gruppe der grossen Erythroblasten mit einer Kernfläche von über 60 mm² Die interphasische RNS Zunahme im Zytoplasma ist bei hämolytischer Anämie und akuter Blutungsanämie grösser, bei der Thalassämie kleiner als in der normalen Erythropoese

Sie ist aber wesentlich geringer als bei hämolytischer Anämie, und auch geringer als in der normalen Erythropoese (Abb 1) Zusammen mit der niedrigen RNS-Bildungsrate ist die Hämoglobinneubildung sowohl im Vergleich zur normalen Erythropoese wie zur hämolytischen Anämie vermindert (Abb 2, verdoppelter Masstab des Hämoglobingehaltes bei Thalassämie!) Auch die Proteinzunahme erfolgt verlangsamt Die kritische Hämoglobinkonzentration der G_2-Phase-Erythroblasten ist noch niedriger als beim hämolytischen Syndrom (Abb 3) Sie wird sowohl in grossen wie kleinen Erythroblasten erreicht Im Gegensatz zur normalen Erythropoese findet auch in den ausreifenden Normoblasten keine nennenswerte Zunahme des Hämoglobingehaltes mehr statt

Akute Blutungsanämie. Die interphasische Zytoplasma-RNS-Zunahme ist noch ausgeprägter als bei hämolytischer Anämie gesteigert (Abb 1) Die Hämoglobinneubildung entspricht ebenso wie die gesamte Proteinzunahme im Ablauf der Interphase den Befunden der normalen

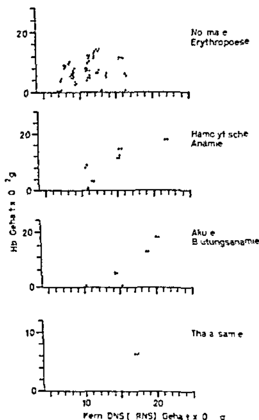


Abb 2 Zunahme des Hamoglobingehaltes in den grossen Erythroblasten (Kernfläche über 60 μm^2) im Ablauf der interphasischen Kern DNS-Synthese Die interphasische Hämoglobinzunahme ist bei Thalassämie am geringsten (Beachte geänderten Massstab)

Erythropoese (Abb 2) Die kritische Hamoglobinkonzentration die in G_2 Phase Erythroblasten erreicht wird ist niedriger als in der normalen Erythropoese (Abb 3) Die kritische Hämoglobinkonzentration wird ähnlich wie bei hämolytischer Anamie und β Thalassämie zum Teil bereits *im jungen Erythroblasten erreicht Die weitere Hämoglobinzu*nahme in den ausreifenden Erythroblasten bis zur Kernausstossung ist geringer als in der normalen Erythropoese und auch bei hamolytischer Anämie aber ausgeprägter als bei β Thalassämie

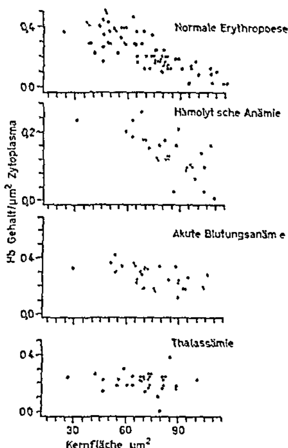


Abb 3 Hämoglobingehalt/µm² Zytoplasmafläche in verschieden grossen Erythroblasten in G_1 Phase Die höchste in G_2 Phase Erythroblasten vor der Mitose erreichte Hämoglobinkonzentration liegt sowohl bei hämolytischer Anämie, akuter Blutungsanämie wie Thalassämie niedriger als in der normalen Erythropoese

Diskussion

Der Vergleich der Hämoglobin-, RNS- und Proteinmengen mit der während der Zellreifung abnehmenden Kerngrösse und dem interphasisch ansteigenden DNS-Gehalt gibt Hinweise über die Neubildungsraten dieser Substanzen in verschiedenen Zyklusphasen und Reifungsstadien

Bei der *hämolytischen Anämie* fanden wir nur wenige junge Erythroblasten in der G_1-Phase als Zeichen der beschleunigt einsetzenden DNS-Synthese Dieser Befund kommt regelmässig nach der Gabe von Erythropoietin zur Beobachtung [16, 17] Ob zusätzlich eine Erythropoese-steigernde Wirkung von Erythrozytenhämolysaten ausgeht, wie

SANCHEZ MEDAL und LABARDINI [20] vermuten, lässt sich nicht entscheiden Der Nachweis nur weniger, nahezu hämoglobinfreier grosser aber auch kleinerer Erythroblasten zeigt, dass die Stammzellumwandlung sowohl in grosse als auch in kleinere rote Vorstufen erfolgt, dass aber die Hämoglobinsynthese schnell einsetzt.

Der RNS-Einstrom in das Zytoplasma ist in der Gruppe der grossen Erythroblasten gegenüber der normalen Erythropoese gesteigert. Das entspricht der vermehrten RNS-Neubildung, die mit der Umwandlung differenzierter Stammzellen in rote Vorstufen unter Erythropoietin einsetzt [9, 10, 18] Obwohl damit die zur Produktion neuer Zellproteine [6, 10] erforderliche RNS in reicher Menge gebildet ist, um über die Aktivierung der Fermente der Hämsynthese [14, 15] die Hb-Synthese in Gang zu setzen, ist nach unseren Befunden die Hämoglobinzunahme im Ablauf der Interphase im Vergleich zur normalen Erythropoese nicht gesteigert, sondern eher herabgesetzt. Die verminderte Hämproduktion wäre durch ein unzureichendes Eisenangebot (Sideroblastenanteil bei dem untersuchten Patienten auf 10% erniedrigt) erklärbar Steht aber Häm nicht in ausreichender Menge zur Verfügung so wird nach MAXWELL *et al* [11] die Bildung eines Inhibitors der Globulinsynthese nicht blockiert, so dass die Protein und damit Hämoglobinsynthese herabgesetzt ist

Die kritische Hämoglobinkonzentration liegt in den G_1 Phase Erythroblasten niedriger als in der normalen Erythropoese Sie wird zum Teil bereits in jungen Erythroblasten erreicht Diese Zellen machen nach erfolgter Mitose keine weiteren Teilungen mehr durch und reifen nur noch aus Deshalb ist die Zahl der durchschnittlich durchlaufenen Reifungsstufen bei hämolytischer Anämie niedriger als in der normalen Erythropoese Da auch die Hämoglobinzunahme in den nur noch ausreifenden Erythroblasten im Vergleich zur normalen Erythropoese herabgesetzt ist muss man eine Hämoglobinsynthese auch noch in den Retikulozyten annehmen, denn der mittlere Hämoglobingehalt der reifen Erythrozyten ist nahezu normal Die breite Streuung des Hämoglobingehaltes und die Anisozytose der Erythrozyten erklären sich durch ihre Entstehung aus verschieden grossen Erythroblasten

Bei der *β-Thalassämie* sind die Befunde durch die Synthesestörung der β Ketten geprägt. Nach BANK und MARK [3] liegt die Störung im β Ketten m RNS-Ribosomenkomplex Die t RNS ist normal Nach ANDERSON [2] ist entweder die β Ketten m RNS in ihrer Sequenz verändert oder sie fehlt ganz. Tatsächlich konnten wir in den jüngsten Erythroblasten eine RNS Zunahme im Zyklusablauf feststellen die geringer als in

der normalen Erythropoese ist. Jedoch ist die Hämoglobinneubildung beginnend in den jüngsten Erythroblasten, noch wesentlich stärker herabgesetzt, so dass das für die einzelnen Zellen errechnete Verhältnis RNS/Hämoglobin höher als in der normalen Erythropoese ist. Die kritische Hämoglobinkonzentration liegt in den G_1-Phase-Erythroblasten etwa in gleicher Höhe wie beim hämolytischen Syndrom, wird aber noch häufiger bereits in jungen (grossen) Erythroblasten erreicht. Da diese Zellen nach durchlaufener Mitose keine weitere DNS-Synthese und Teilung mehr durchlaufen, müssten die Erythrozyten, die nach Kernausstossung aus besonders grossen Erythroblasten entstehen, ein hohes Volumen aufweisen. Da wir aber ein mittleres Erythrozytenvolumen von nur 70 μm^3 bestimmen konnten, muss man schliessen, dass der grösste Teil dieser roten Vorstufen nicht zur Ausreifung gelangt, sondern verfrüht zugrunde geht. Die Befunde wiesen damit gleichzeitig auf das Vorliegen einer ineffektiven Erythropoese bei Thalassämie hin. Einen Anhalt für eine vermehrte Zahl an Reifungsstufen, wie STOHLMANN [21] vermutet, konnten wir dagegen nicht gewinnen.

Bei akuter Blutungsanämie gleichen die Befunde denjenigen bei hämolytischer Anämie. Karyometrisch fehlen die kleinen Erythroblasten. Es finden sich vermehrt Erythroblasten mit hohem Kern-DNS-Gehalt. Der RNS-Einstrom in das Zytoplasma ist während der Interphase der jüngsten (grössten) Erythroblasten noch ausgeprägter als bei dem Kranken mit hämolytischer Anämie. Die kritische Hämoglobinkonzentration liegt nicht so niedrig wie bei der hämolytischen Anämie, wird aber ebenfalls bereits in jungen Erythroblasten erreicht. Dieser Befund weist auf eine verminderte Zahl von Reifungsstufen auch bei der akuten Blutungsanämie hin. Eine verminderte Zahl an Reifungsstufen konnte im Tierversuch erstmals von ALPEN und CRANMORE [1] nachgewiesen werden. Ein Einfluss des Eisenverlustes durch die Blutung ist zu diesem Zeitpunkt noch nicht nachweisbar.

Nach DUKES [6] und ORLIC *et al.* [17] sowie POWSNER und BERMAN [19] und DUKES [7] ist eine gesteigerte RNS- und DNS-Synthese durch Erythropoietin auszulösen. Da anderseits nach akutem Blutverlust wie bei hämolytischem Syndrom mit vermehrter Erythropoitinwirkung zu rechnen ist (Übersicht bei REMMELE [23]) dürften die von uns bei stimulierter Erythropoese beobachteten Befunde hiermit im Zusammenhang stehen.

Die gesteigerte (?) Umwandlung von Stammzellen in rote Vorstufen erfolgt in einer kurzen G_1-Phase nicht nur zu grossen sondern auch zu kleinen Erythroblasten. Die kritische Hämoglobinkonzentration, die

nach STOHLMANN *et al* [22] die weitere DNS-Synthese stoppt, liegt niedriger und wird zum Teil bereits in jungen Erythroblasten erreicht. Konnten wir schon für die normale Erythropoese zeigen, dass die Zahl der durchlaufenen Reifungsstufen nicht konstant ist, so beweisen die vorliegenden Befunde, dass sowohl bei hämolytischer Anämie, β-Thalassämie wie Blutungsanämie die Zahl der durchlaufenen Reifungsstufen vermindert ist.

Zusammenfassung

Die Stammzellumwandlung zu morphologisch erkennbaren roten Vorstufen erfolgt bei hämolytischer Anämie, akuter Blutungsanämie und β-Thalassämie während einer kurzen G_1-Phase zum Teil bereits zu kleineren Erythroblasten. Der interphasische RNS-Einstrom ins Zytoplasma ist bei hämolytischer Anämie und Blutungsanämie gesteigert, bei β-Thalassämie herabgesetzt. Die interphasische Hämoglobinneubildung erfolgt in jungen Erythroblasten bei hämolytischer Anämie und akuter Blutungsanämie wie in der normalen Erythropoese, sie ist bei der Thalassämie auf die Hälfte erniedrigt. Die «kritische Hämoglobinkonzentration» liegt in den untersuchten Fällen in den G_1-Phase-Erythroblasten niedriger als in der normalen Erythropoese und wird gehäuft in jungen Erythroblasten erreicht. Die Zahl der Reifungsstufen ist daher herabgesetzt.

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Lipid Peroxidation in Erythrocytes

Supravital Staining of Peroxidised Cells by Crystal Violet

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Abstract Supravital staining with crystal violet of normal erythrocytes which had been exposed to hydrogen peroxide vapour, resulted in the appearance not only of Heinz bodies but also in an overall blue coloration of a proportion of the erythrocytes Inhibition of catalase by aminotriazole or by sodium azide greatly increased the proportion of such 'blue cells A close relationship was found between the percentage of blue cells and the degree of lipid peroxidation as measured by malonyldialdehyde formation

Key Words
Blue cells
Crystal violet
Erythrocyte lipids
Heinz bodies
Lipid peroxidation
Oxidation in erythrocytes

Crystal violet has long been in use as a stain for Heinz bodies, the insoluble precipitated products of haemoglobin breakdown, formed in the erythrocyte as a result of oxidant stress, often in association with enzymopathies or haemoglobinopathies A number of drugs including phenylhydrazine and antimalarials of the 8-aminoquinoline group exert an oxidant effect on the erythrocyte as a result of the intracellular production of hydrogen peroxide [4–6]

While studying the effect of hydrogen peroxide vapour in forming Heinz bodies in erythrocytes, subsequently stained supravitally with crystal violet, we noted that some of the cells stained in an overall blue manner, such cells we have termed 'blue cells' There appears to be no mention of this phenomenon in the literature We have investigated the formation of blue cells' in erythrocyte suspensions subjected to hydrogen peroxide vapour in the presence of enzyme inhibiting drugs, and in

[1] With the technical assistance of Joan H Smith

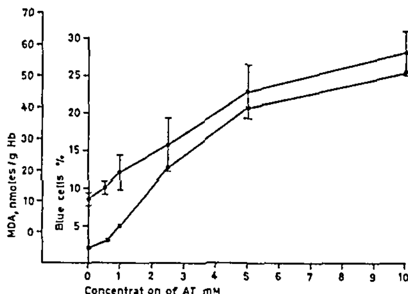


Fig 1 MDA and blue cell formation in erythrocytes after peroxidation in the presence of AT ■ = Blue cells, ● = MDA±SD

tors, sodium azide and AT, there were only 1–5% blue cells formed in the stained erythrocyte suspensions and only a small amount of MDA was produced (10 7 ± 1 9 nmoles/g Hb)

A Cells exposed to H_2O_2 in the presence of AT In Conway units containing a range of concentrations of AT, erythrocytes from 6 normal persons were subjected to H_2O_2 vapour for 3 h at 37 °C. With increasing concentrations of AT, there were progressive increases in the amounts of MDA and of blue cells formed (fig 1) and in the degree of inhibition of the activities of catalase and glutathione peroxidase (fig 2) No Heinz bodies were formed in any of the preparations of cells exposed to H_2O_2 and AT

B Cells exposed to H_2O_2 in the presence of azide The mean results for the formation of MDA and blue cells in 8 normal subjects are shown in figure 3, the mean results for catalase inhibition are also shown With increasing concentrations of azide there were increasing amounts of MDA and of blue cells formed, and progressive reduction in the activity of catalase In addition to the blue cells, with 10 mM azide, between 7 and 40% of the erythrocytes contained Heinz bodies (fig 4)

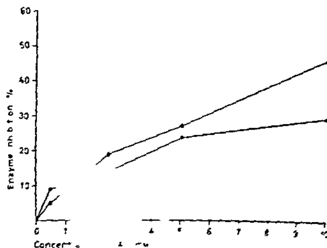


Fig 2 Activity of ca se and G erythrocytes after peroxidation in the presence of AT ● = SH Px ▲ e

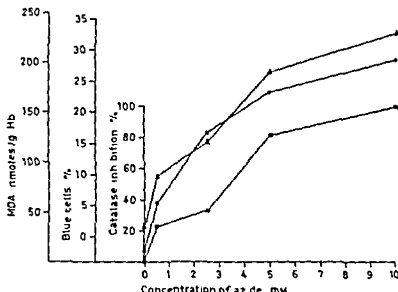


Fig 3 MDA and blue cell formation, and the activity of catalase in erythrocytes after peroxidation in the presence of azide ● = MDA, ▲ = blue cells ■ = catalase

Spontaneous haemolysis No relationship existed between the percentage of blue cells and the degree of spontaneous haemolysis of erythrocytes after peroxidation in the presence of AT Spontaneous haemolysis was less than 1% even when 22% of the erythrocytes stained as blue cells

Mechanical fragility The mechanical fragility of erythrocytes exposed to hydrogen peroxide in the above experiments, with or without the addition of AT in concentrations up to 10 mM, was less than 6%. the formation of blue cells bore no relationship to the degree of mechanical fragility

Site of staining in blue cells An erythrocyte suspension was peroxidised in the presence of 10 mM AT for 3 h at 37 °C, and the following experiments were performed One portion of the suspension was stained with crystal violet Part of this was used to prepare a smear (a), and part was haemolysed with distilled water, the membranes were spun down and a smear made of the deposit (b) Another portion of unstained suspension was haemolysed with distilled water, spun at 12,000 g for 10 min and the supernatant discarded The membrane material was washed with water, spun down again, stained with crystal violet and a

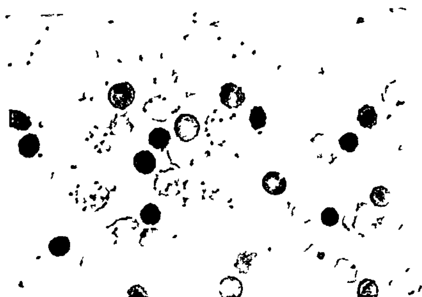


Fig 4 Erythrocytes staining as blue cells after peroxidation in the presence of azide (10 mM). Many other erythrocytes contain Heinz bodies

smear prepared (c). Typical blue cells were present in the preparation (a) and a proportion of the ghosts in preparations (b) and (c) showed purple grey overall staining. From this it was concluded that the site of staining of the blue cells was the erythrocyte membrane

Discussion

The erythrocyte is subjected to a variety of both physical and chemical stresses during its lifespan. These include the mechanical trauma experienced by cells during circulation and oxidative stresses due to oxygen or to infections or to hydrogen peroxide. Hydrogen peroxide may be produced from the autoxidation of haemoglobin or by the oxidation of certain drugs and their metabolites [4–6, 28] or as a product of normal cell metabolism. The erythrocyte is in possession of a variety of factors protecting against oxidant stress; these include the H_2O_2 detoxifying enzymes catalase and GSH Px and certain antioxidants

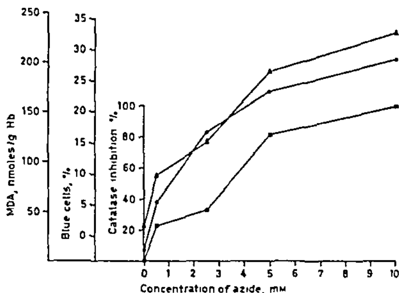


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tion as a measure of lipid peroxidation in blood from such patients is at present under investigation

The results of the present study show that the formation of MDA is accompanied by a change in the erythrocyte membrane causing an overall blue colour when stained supravitally with crystal violet It is apparent that the formation of blue cells, besides being related to MDA formation, is related to reduced activities of both catalase and GSH-Px From the results in the experiments with maleimide, however, it appears that GSH-Px does not play a major role in protecting the erythrocytes against the high concentrations of hydrogen peroxide vapour in this system It is generally believed that GSH-Px normally functions to remove the small quantities of hydrogen peroxide that are produced in the erythrocyte as a result of normal metabolism and from the action of certain drugs [3, 13, 18] The value of blue cell counts as an index of lipid peroxidation is clearly of potential importance in studying the role of oxidant drugs in causing or enhancing lipid peroxidation, it is possible that observations on blue cells may be of particular value in studying the effect of drugs which interfere with the spectrophotometric measurement of MDA

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Table I

Substances added to red cells	MDA nmoles/g Hb	Blue cells, %
None	10 0	2
NEM	14 2	4
AT	59 5	24
NEM+AT	60 9	26
Azide	123 3	31
Azide+NEM	117 5	25

Final concentrations AT, 10 mM azide, 5 mM NEM, 0 5 mM Erythrocyte suspensions contained 4 g% Hb and the total volume of the mixture in the outer well was 3 5 ml Results are the mean of 2 experiments

notably tocopherol [8, 13, 14] Dietary selenium may also be involved in protecting the erythrocyte against oxidant stress [22]

Investigations of the *in vitro* effect of oxidant stress on the erythrocyte have generally been carried out in the absence of protecting factors i e, the enzymes were inhibited, blood from tocopherol-deficient subjects was studied, and high concentrations of hydrogen peroxide were employed *Such conditions are necessarily unphysiological, but the results* have shown that in erythrocytes peroxidised by H_2O_2 there is, particularly in the presence of azide, considerable formation of the lipid breakdown product MDA [2, 8, 23–25] MDA is derived mainly from unsaturated fatty acids containing 3 or 4 unsaturated bonds, such as arachidonate and linolenate [7] As these represent only a portion of total unsaturated fatty acids in the erythrocyte, MDA formation represents only a portion of total peroxidised unsaturated fatty acids, however, its formation is generally accepted as a measure of lipid peroxidation [17, 23, 29, 30]

MDA formation during peroxidation of the erythrocyte has been shown to be increased in abetalipoproteinaemia [8], autoimmune haemolytic anaemia, G6PD deficiency, thalassaemia major [23] and tocopherol deficiency [2, 8] Low plasma levels of tocopherol are common in infants particularly prematures and those fed on a diet rich in polyunsaturated fatty acids, in such instances anaemia may develop [19, 21] In adults, low plasma levels of tocopherol have been found in patients with malabsorption [1, 11, 15, 16] The study of blue cell and MDA forma-

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It was subsequently shown that the prothrombin content and activity in the plasma of patients with the Friuli disorder is normal [12]. Furthermore, it was demonstrated that the cross-over electrophoresis mobility of factor X Friuli is identical to that of normal factor X and is different from the mobility shown by the coumarin induced abnormal factor X [13, 16]. Finally, it was demonstrated that no PIVKA (prothrombin inhibitor vitamin K absence) type inhibitor is present in Friuli plasma [17].

Since all homozygote patients originally described were born in an isolated valley of the Italian northeastern region called Friuli, the eponym 'Friuli' was attached to the condition [7]. Recently, an additional patient with a different geographical background was described [14]. Several heterozygotes have been described together with these 11 homozygote patients. However, only fragmentary data have been reported so far about the latter patients. The object of this paper is to report in detail data concerning the 57 heterozygote studied by us so far. This is the largest number of heterozygotes for any prothrombin complex factor deficiency or abnormality ever studied.

Material and Methods

Material and coagulation methods have been reported in detail elsewhere [6, 7, 10]. Only new data will be given herein. We used deep frozen plasma of a patient with severe classical factor X deficiencies [9]. Rabbit brain and lung thromboplastin was supplied by Warner Chilcott Laboratories, Morris Plain, N.J. Human brain thromboplastin was kindly supplied by Dr. L. POLLER, Withington Hospital, Manchester, England. Human placenta thromboplastin was supplied by Behringwerke, Marburg, FRG. Anti-human factor X rabbit antiserum was kindly supplied in lyophilized form by Dr. D. ARONSON, National Institutes of Health (NIH), Bethesda, Md., [1, 4].

The plasma of the 57 subjects found so far to be heterozygotes for the abnormality have been studied. 39 of these patients were members of the families of our 10 homozygote patients born in Friuli. The remaining 18 patients were not related to any of our homozygote patients, but 16 of them were born in the valley where the 10 homozygotes were discovered (fig. 1, 2). Two other heterozygotes taken into consideration belonged to the family of the only homozygote patient with this disorder so far described outside Friuli. 29 patients were male, 28 were females. Ages varied between 6 and 70 years.

77 normal subjects were also studied. They were composed of two groups: 46 subjects were relatives of our homozygote patients. The remaining 31 cases were normal subjects of both sexes whose plasma yielded a normal prothrombin time on a routine coagulation screening. The prothrombin times were carried out in all instances on fresh plasmas. The factor X assay was carried out either on fresh or on

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Abnormal Factor X (Factor X Friuli) Coagulation Disorder. The Heterozygote Population

A Study of 57 Subjects

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Key Words
Abnormal factor X
Bleeding disorders
Blood coagulation
Factor X Friuli

Abstract Data are reported pertaining to the 57 subjects so far found to be heterozygotes for the abnormal factor X (factor X Friuli) coagulation disorder 41 of these subjects were related to the 11 patients known to be homozygote for the defect Their average factor X level was 59 7% The difference between such an average and those found in the homozygote population (10 9%) and in two normal groups (106 and 103%) was statistically significant The heterozygote usually presents a 1 to 2.5 sec prolongation of the prothrombin time A significant negative correlation was found to exist between the prothrombin time prolongation in seconds and the percentile factor X level Factor X in the heterozygote population results to be lower when assayed using human brain or human placenta thromboplastins as compared with a rabbit brain and lung thromboplastin

The cross over electrophoresis mobility of heterozygote Friuli plasma is identical to that of homozygote Friuli plasma and normal plasma About one third of the heterozygote patients presented bleeding manifestations

Classical factor X deficiency was first identified in 1956–57 [2, 21, 25] A bleeding disorder due to an abnormal factor X was described by us in 1969 and 1970 [5–7] This 'peculiar factor X may only be activated very slowly by whole or partial tissue thromboplastin, whereas it may still be normally activated by Russell s viper venom Consequently, the prothrombin and the partial thromboplastin times are prolonged in these patients whereas the Stypven-cephalin clotting time is normal [5–7, 10] On the other hand in classical factor X deficiency, all these tests are prolonged

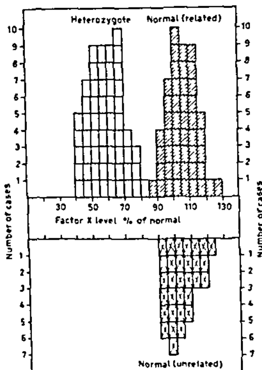


Fig 3 Distribution of factor X levels in 57 heterozygotes and in 77 normal subjects The normal subjects are divided in two groups (1) subjects related to our 11 homozygote patients, and (2) subjects unrelated to our homozygote patients. The factor X level reported for the heterozygotes was often the average of more than one determination The tissue thromboplastin used in these determinations was always the same (rabbit brain and lung) The substrate was charcoal filtered ox plasma

level found in a heterozygote was 82% of normal whereas the lowest level found in the two normal groups was 88 and 90% of normal respectively

The lowest level observed in the heterozygote population was 42%. The statistical analysis of results showed that the difference between the averages observed in the normal groups and in the homozygote group was significant ($p<0.001$) The same was true for the differences observed between the heterozygote and the two normal groups ($p<0.001$) and between the heterozygote and the homozygote groups ($p<0.001$) On the

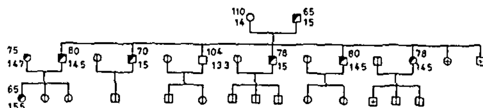


Fig 1 Pedigree of a family with several heterozygotes Interestingly one of the heterozygotes married another heterozygote Only 1 of the 3 children resulting from this mating was studied and found to be heterozygote too The other 2 children were not available for study but were asymptomatic The lower numbers show the prothrombin time in seconds The upper numbers refer to the factor X level in per cent ◩ or ◐ = Heterozygote asymptomatic or mildly symptomatic, □ or ○ = studied, normal, ⊞ or ⦶ = not studied, asymptomatic, normal?

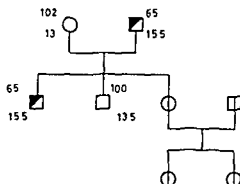


Fig 2 Pedigree of another family The symbols and numbers have the same meaning as in figure 1

deep frozen plasmas Diagen charcoal filtered ox plasma was used sa substrate, and rabbit brain and lung human placenta or human brain extracts as thromboplastins

Cross over electrophoresis or electrosyneresis was carried out in agar according to a previously reported modification [13] of the method proposed by BUSSARD [3]

Results

The results are summarized in table I and in figure 3 The average of the factor X levels obtained in the homozygote population was 10 9% The average levels found in the heterozygote population and in the two normal groups were 59 7, 106 0 and 103 0%, respectively The highest

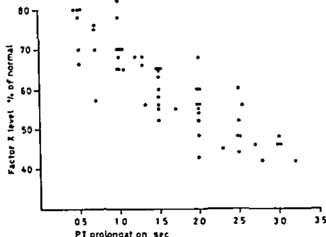


Fig 4 Correlation between prothrombin time (PT) prolongation and factor X levels observed in the heterozygote population The correlation was proved to be statistically significant $r = -0.85$ $t = 12.1$ $p = <0.001$

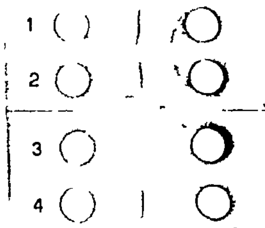


Fig 5 Cross-over electrophoresis (electrosyneresis) of (1) normal plasma, (2) heterozygote Friuli plasma (3) plasma of a patient with classical factor X deficiency and (4) homozygote Friuli plasma A main or factor X band is evident in 1 2, and 4 no difference in the migration of the band is noted Disregard the lighter bands which owe to secondary activities of the antiserum used

Table II Bleeding manifestations in the heterozygote population (a total of 22 patients could be considered mildly symptomatic)

Bleeding manifestation	Number of patients		Percentile figure
	considered	affected[1]	
Easy bruising	57	4	7
Epistaxis	57	4	7
Spontaneous bleeding from the gums	57	2	4
Excessive bleeding after tooth extractions	53	14	26
Menorrhagia	26	1	4
Excessive bleeding after parturition	22	3	13

[1] Some subjects presented more than one bleeding manifestation

ing symptoms (38%) Bleeding was always mild, and a blood transfusion was given only in one instance after a tooth extraction The commonest complaint was excessive bleeding after tooth extractions

Discussion

The population of the valley where the 10 patients with this peculiar disorder were first discovered is about 4,000 people. This is an isolated valley which only in recent years has seen a change in the traditional life pattern. Marriages among relatives were common practice until a few decades ago This phenomenon is well documented by the f... at only about 10 last names enco... 60% of the popu... to these geographical and ... conditions it may ... assumed that the original m... for the defect ... curred at the heterozygo... became manif... gote could have appeared ... as a result of two ... heterozygotes ... is in fact ... co... in type ... classical cie...

Table III Factor X levels in % as determined using different thromboplastin preparations in two groups of patients heterozygote for the abnormal factor X coagulation disorder

Patient No	Thromboplastin obtained from rabbit lung and brain[1]	Thromboplastin obtained from human placenta[2]
1	82	72
2	60	58
3	68	64
4	68	52
5	68	45
6	65	60
7	66	50
8	66	55
9	65	60
		Manchester human brain thromboplastin
1	70	68
2	60	56
3	70	68
4	64	58
5	70	56
6	60	52
7	68	56
8	75	68
9	67	60
10	72	66
11	80	66
12	78	64
13	78	66
14	78	54
15	80	68
16	70	68
17	52	50
18	48	40

[1] Simplastin, Warner-Chilcott
[2] Calcium-thromboplastin Behringwerke

The report of family pedigrees with only heterozygotes is of interest (fig 1, 2) It confirms the correctness of our interpretation of the hereditary transmission of this disorder Unless a heterozygote or carrier mar-ries another heterozygote no homozygote may result from the mating

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The population of the valley where the 10 patients with this peculiar disorder were first discovered is about 4,000 people This is an isolated valley which only in recent years has seen a change in the traditional life pattern Marriages among relatives were common practice until a few decades ago This phenomenon is well documented by the fact that only about 10 last names encompass at least 60% of the population Due to these geographical and socio-economic conditions it may be safely assumed that the original mutation responsible for the defect might have occurred at the heterozygote level Once this became manifest, a homozygote could have appeared in a few generations as a result of the mating of two related heterozygotes The hereditary pattern is in fact autosomal incompletely recessive in type The same is true for classical factor X deficiency [20, 22]

ozygotes for factor VII deficiency seem to be asymptomatic [19] The frequence of bleeding manifestations found in our heterozygote population may not be the real one So far, only symptomatic patients and their relatives have been studied, and therefore, we have probably introduced a bias into the selection of cases

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Hereditary Elliptocytosis Associated with Pernicious .

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Abstract A patient with hereditary elliptocytosis and pernicious anaemia is described After the treatment with hydroxocobalamin the patient s clinical condition and blood picture became entirely normal except the persistence of elliptocytes of hereditary origin Gene interaction was not encountered

Key Words
Elliptocytosis
Pernicious anaemia

Hereditary elliptocytosis is an uncommon disorder, but not rare in Northern Europe It is a genetic morphological anomaly of red cells and has been described in association with other genetic disorders, including HbS [7, 11], β-thalassaemia [1, 3, 5 8, 10] HbC [2] hereditary telangiectasia [9] and acquired autoimmune haemolytic anaemia [6] The present patient is a first reported case with combined hereditary elliptocytosis and pernicious anaemia

Case Report

A 76-year-old lady presented with a 2 year history of increasing tiredness irritability and a recent development of paraesthesia in her fingers and toes Physical examination revealed severe anaemia smooth sore tongue Liver and spleen were not enlarged Neurological examination showed diminished pinprick touch sensation over the extremities and depressed ankle and knee jerks and no other abnormality

Haemoglobin 7 1 g/100 ml PCV 22% MCHC 32% MCV 138 μm^3 RBC 1 62 mill/mm³, WBC 7,500 mm³ normal differential count Reticulocyte 6% Platelet count 190 000 mm³ ESR 4 mm/h Blood film (fig 1) showed large number of rod shaped elliptocytes (approximately 50%) macroovalocytes Howell Jolly bodies and hypersegmented neutrophils Red cell fragility was slightly increased

Acta Haemat. 51: 51–54 (1974)

Hereditary Elliptocytosis Associated with Pernicious Anaemia

M. L. Ghosh

Department of Haematology, District General Hospital, Barnsley

Key Words
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Abstract. A patient with hereditary elliptocytosis and pernicious anaemia is described. After the treatment with hydroxocobalamin the patient's clinical condition and blood picture became entirely normal except the persistence of elliptocytes of hereditary origin. Gene interaction was not encountered.

Hereditary elliptocytosis is an uncommon disorder, but not rare in Northern Europe. It is a genetic morphological anomaly of red cells and has been described in association with other genetic disorders, including HbS [7, 11], β thalassaemia [1, 3, 5, 8, 10], HbC [2], hereditary telangiectasia [9] and acquired autoimmune haemolytic anaemia [6]. The present patient is a first reported case with combined hereditary elliptocytosis and pernicious anaemia.

Case Report

A 7[illegible]-year-old lady presented with a 2-year history of increasing tiredness, irritability and a recent development of paraesthesia in her fingers and toes. Physical examination revealed severe anaemia, smooth sore tongue. Liver and spleen were not enlarged. Neurological examination showed diminished pinprick, touch sensation over the extremities and depressed ankle and knee jerks and no other abnormality.

Haemoglobin [illegible] g/100 ml; PCV 22%; MCHC 32%; MCV 138 µm³; RBC 1.62 mill/mm³; WBC 7,500/mm³; normal differential count. Reticulocyte [illegible]%. Platelet count 190,000/mm³. ESR 4 mm/h. Blood film (fig. 1) showed large number of rod-shaped elliptocytes (approximately 50%), macroovalocytes, Howell-Jolly bodies and hypersegmented neutrophils. Red cell fragility was slightly increased



Fig 1 Blood film before vitamin B_{12} therapy showing macrocytes and hereditary elliptocytes × 600

Fig 2 Blood film 3 months after vitamin B_{12} therapy showing numerous typical rod shaped hereditary elliptocytes × 600

Bone marrow examination showed cellular marrow with typical megaloblastic erythropoiesis and many giant metamyelocytes. Iron stain showed increased extracellular and reticulin iron.

Her blood group is O Rh, genotype cDE/cde. Serum bilirubin 2 mg%. Serum B_{12} 60 pg/ml. Serum folate 13 ng/ml. Serum iron 170 μg/100 ml. Total iron binding capacity 320 μg/100 ml. Her serum contained gastric parietal cell antibody, but no intrinsic factor antibody was present. Gastric analysis after pentagastrin stimulation demonstrated achlorhydria. Schilling test without and with intrinsic factor confirmed loss of intrinsic factor secretion consistent with pernicious anaemia.

She was treated with intramuscular hydroxycobalamin with reticulocyte response up to 30%. Her general condition improved and after 3 months of treatment the neurological symptoms disappeared and her haemoglobin increased to 12 5 g/100 ml, last reticulocyte count 1%, and the blood film (fig 2) showed marked elliptocytosis but no macrocyte was present.

Discussion

Oval or elliptical red cells may be present in megaloblastic anaemia, iron deficiency anaemia and myelosclerosis but there is no evidence of familial incidence. The blood film in a typical case with hereditary elliptocytosis with up to 90% oval or elongated red cells and inconspicuous poikilocytes, microcytes and cell fragments except in patients with increased haemolysis [4] and the positive family history confirms the diagnosis. Although family study was not possible in the present case, but such a high degree (about 70%) of typical rod-shaped elliptocytes cannot be explained due to pernicious anaemia. Further, this is borne out by the fact that after the haemoglobin reached a normal level (12 5 g/100 ml) with vitamin B_{12} therapy the elliptocytosis not only persisted but was exaggerated confirming the presence of hereditary elliptocytosis.

The macrocytic anaemia with megaloblastic bone marrow, low serum B_{12} level, lack of intrinsic factor, achlorhydria after maximum stimulation with pentagastrin and complete haematological response to B_{12} therapy being the hallmarks of pernicious anaemia have been demonstrated clearly in this present patient.

The exact relationship between hereditary elliptocytosis with probable genetic defect of red cell membrane and pernicious anaemia which is generally agreed as autoimmune disease in this present case is not clear. Genetic studies have shown that hereditary elliptocytosis is an autosomal dominant inheritance and the propensity of gastric lesion causing development of parietal cell antibody in pernicious anaemia appears to be au-

tosomal dominant trait with variable expression On the other hand hereditary elliptocytosis is present shortly after birth whereas the peak incidence of pernicious anaemia is at the age of 60 Furthermore the exact hereditary factor and its mode of transmission in pernicious anaemia is unknown

PERILLIE and CHERNOFF [10] noted the evidence of increased haemolysis due to summation of clinical effect of the hereditary elliptocytosis and β thalassaemia genes in the same patient similar to that described by DE VRIES *et al* [5] There seems to be no evidence to suggest mutual enhancement of the involved genes for hereditary elliptocytosis and pernicious anaemia in this present case It is worth mentioning that the present observation confirms the similar findings by others in cases presenting with hereditary elliptocytosis associated with β thalassaemia [1, 8] *Hb S [11] and Hb C [2]*

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Acta Haemat 51 55–62 (1974)

Purpura Characterised by Thrombasthenia Associated with Alterations of Blood Lipids[1]

D Del Principe, G Ballati, M Castro, G Digilio and O Giardini

Pediatric Clinics I (Director Prof A Colarizi) and II (Director Prof E. Rezza), University of Rome, Rome

Abstract A case is reported of primary hemorrhagic syndrome produced by a functional defect of platelets due to an extrinsic cause, being associated with a decrease of all blood lipid fractions particularly of phosphatidylcholine and with an absence of phosphatidyl ethanolamine The possible relationships between the two alterations are discussed

Key Words
Bleeding disorders
Blood lipids
Lipoproteins
Platelet functions
Purpura
Thrombopathies

According to Nilsson and Crolberg [19], primary hemorrhagic syndromes characterized by prolonged bleeding time and by a normal number of platelets, can be divided into two groups In the first, the defect is intrinsic to the platelets, in the second the alteration of platelet function is correlated to a more or less known plasmatic defect (von Willebrand's disease, Rita's disease) Disturbances of platelet function may also be associated with various genetic and congenital conditions, such as diseases of connective tissue [9] glycogenosis [11, 20], Niemann-Pick disease [8], afibrinogenemia [13] and heart diseases [17]

In the present paper a hemorrhagic syndrome is reported, in which, besides a functional alteration of platelets due to an extrinsic defect, a decrease of the various lipid fractions in the serum was present.

Methods

All serum tests were performed upon venous blood collected after a 12 hour fasting Bleeding time was determined by Duke's method carried out upon both ears (normal 1–4 min)

[1] We are grateful to Miss [illegible] for her technical aid

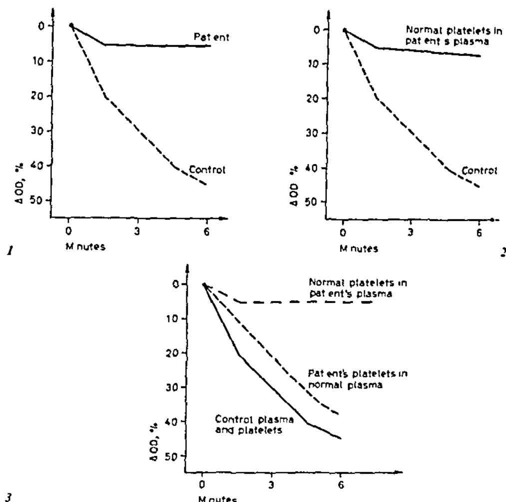


Fig 1 Platelet aggregation following ADP addition (200 μg/ml) in the patient and in one control

Fig 2 Platelet aggregation after addition of norepinephrine (1 μg/ml) in the patient and in a normal control

Fig 3 Aggregation after ADP addition of normal platelets suspended in the patient's plasma and of patient's platelets suspended in a normal plasma

Discussion

The results obtained in the studied case reveal (1) a decreased aggregation of platelets, not due to any intrinsic cause, since they would respond normally to ADP addition, if they were suspended in a normal plasma, (2) *the presence in the patient's plasma of an inhibiting action*

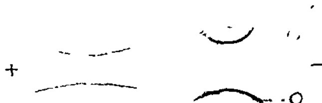


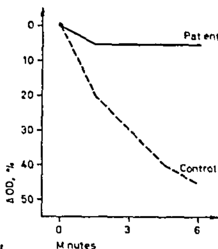
Fig 4 Immunoelectrophoretic examination of lipoproteins Above patient's plasma, below pool of normal sera Antihuman plasma protein serum has been placed in the central groove Staining with oil red O A decrease of the patient's lipoproteins is noted as compared to normal controls

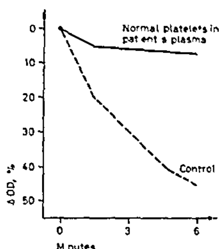
Table I Lipid and phospholipid fractions in the patient's serum (mg 100 ml of serum)

	Patient	Normal values
Total lipids	150	510
Total cholesterol	63	196
Triglycerides	16	92
Total phospholipids	66	222
Sphingomyelin	23	46
Phosphatidylcholine	43	155
Phosphatidylserine	absent	absent
Phosphatidylethanolamine	absent	9

upon platelet aggregation, since normal platelets suspended in the patient's plasma would lose their sensitivity to ADP This phenomenon was also present when the ADP was incubated in the patients platelet-free plasma prior to the addition of normal platelets, and (3) a dyslipidemia featuring a reduction of all serum lipid and phospholipid *fractions, particularly* of phosphatidylcholine, with absence of phosphatidylethanolamine

The physical examination of the patient as well as the other investigations have ruled out the above mentioned diseases, which may be accompanied by functional alterations of thrombocytes The most common causes of extrinsic alterations of platelet aggregation, such as a deficiency of coagulation factors (fibrinogen, calcium) the presence of macroglobu-





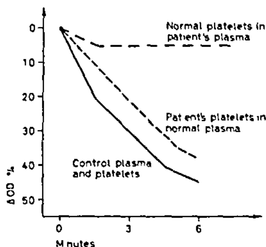


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Fig 3 Aggregation, after ADP addition, of normal platelets suspended in the patient's plasma and of patient's platelets suspended in a normal plasma

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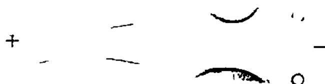


Fig 4 Immu... phoretic examination of lipoproteins Above patient s plasma below p... rmal sera Antihuman plasma protein serum has been placed in the cent... Staining with oil red O A decrease of the patient s li...poproteins is note... ed to normal controls

Table I Lipid and ... fractions in the patient s serum (mg 100 ml of serum)

	Patient	Normal values
T	150	510
T	63	196
Tr	16	92
Tc	66	222
Sp	23	46
Ph	43	155
Ph	absent	absent
Ph ... ityle ... re	absent	9

upon platelet ag... ation sin... ormal platelets suspended in the patients plasma wo... ose their ... tivity to ADP This phenomenon was also present when ... ADP wa... ncubated in the patients platelet free plasma prior to t... ... of ... mal platelets ... featuring a reducti... ...h of phosphati...

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linemia of hyperbilirubinemia or of an uremic syndrome have been ruled out The assumption of a metabolic defect of ADP and AMP is made unlikely by the finding of normal blood levels of these substances

The influence of lipids in general and of lipoproteins in particular upon platelet aggregation has been recently reported by various authors It was observed by MARTINOV and GROMNATSKY [18] that alimentary hyperlipemia and endogenous hyperbetalipoproteinemia may intensify platelet aggregation A platelet aggregating action by fatty acids has been stressed by HASLAM [14] An increased adhesiveness and aggregation of platelets following fatty food intake was also remarked by COCCHERI *et al* [6] FARBISZEWSKI and WOROWSKI [10] studied the effects of purified β lipoproteins upon platelet adhesiveness and aggregation drawing the conclusion that they significantly accelerate the platelet aggregation induced by ADP

Studies by BOLTON *et al* [2] have proved in the plasma of subjects presenting some vascular diesease the existence of a transferable factor *which produces an increased sensitivity of platelets to ADP Such a factor* is supposed to be made up of the phosphatidylcholine contained in low density lipoproteins as well as of a specific labile enzyme which converts phosphatidylcholine into lysophosphatidylcholine This enzyme would only be active upon phospholipids of low density lipoproteins and upon free lecithin

BOLTON s studies might supply an explanation for our own findings in fact in our case the decrease in absolute values of phosphatidylcholine might prevent the normal activity of the specific enzyme thus provoking a decreased formation of lysophosphatidylcholine with a consequent lowering of platelet aggregation

On the other hand in the patient studied by us some other alterations in the composition of serum phospholipids were present such as the absence of phosphatidylethanolamine Moreover platelet aggregation reappeared when the β lipoproteins were removed from the patient s plasma The latter phenomena are difficult to explain It should therefore be necessary to know as it was already noted by BOLTON *et al* [2] not only the lipid composition of lipoproteins but also the physical conformation of the various phospholipids contained in them their composition in fatty acids as well as their capability of being attacked by phospholipases

It seems to us however that our case is worth reporting since it happens to be the first clinical observation which conveys this particular alteration not yet fully known of the mechanism of platelet aggregation

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Ciba Foundation Symposium 13. **Haemopoietic Stem Cells.** Elsevier/North Holland, Amsterdam 1973. 345 pp. Dfl 44.– US $ 15.40.

This symposium, held July 13th–14th 1972 at the Ciba Foundation in London, was organized in honour of Professor JOSEPH YOFFEY. Authors of international reputation presented and discussed thirteen papers dealing with the stem cell role of the lymphocyte transitional cell, the morphological identification of stem cells, the experimental models for studying haemopoietic stem cells, the properties of lymphocytes and lymphoid cells in the bone marrow, the cellular communication in haemopoietic differentiation, the control of granulopoiesis and thrombopoiesis, the stem cell compartments after depression of erythropoiesis, the effects of anaemia on DNA-synthesizing cells, and the influence of ageing and malignant tumors. The existence and nature of a primitive precursor cell was thoroughly discussed, and studies were reported which may lead to a new concept of haemopoies[illegible]he book is a most valuable source of information for laboratory investiga[illegible]d clinicians interested in blood cell formation.

H. R. MARTI, *Aarau*

Standard Techniques for the Measurement of Red Cell and Plasma Volume

The expert panel on the Diagnostic Applications of Radioisotopes in Haematology which was established by the International Committee for Standardization in Haematology has prepared a document which deals with the technical and analytical aspects of red cell and plasma volume measurements. After an introduction which deals with general principles and draws attention to the areas of particular difficulty in these methods the document contains the following sections (1) Techniques for the estimation of red cell volume (2) Techniques for the estimation of plasma volume (3) The measurement of packed cell volume (4) The assessment of automated blood volume equipment (5) Sequential blood volume estimations (6) Estimation of total blood volume as the sum of red cell plasma volumes (7) Presentation and analysis of results (8) The radiation dose which the patient receives during these investigations

The purpose of the document is to enable measurements obtained in different centres to be reliably compared with each other The document has been published in Brit J Haemat 25 797 1973 and will also be published in a number of national journals

L. Szur
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L'Association Française des Hemophiles a cree un prix sous le nom de Prix International de l'Association Française des Hemophiles destiné à récompenser les travaux d'un chercheur ou d'une équipe de chercheurs permettant de faire progresser la thérapeutique de la maladie d'en prévenir le développement ou d'en assurer le traitement curatif

Pour tous renseignements s'adresser au Secrétariat de l'Association Française des Hémophiles (Centre National de Transfusion Sanguine) 6 rue Alexandre Cabanel *F 75015 Paris* (France)

Acta Haemat. 51: 65–75 (1974)

Rosette-Forming Lymphocytes in Normals and Patients with Malignant Lymphomas[1]

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Key Words
Ig positive lymphocytes
Lymphocyte populations
Malignant lymphomas
Rosette forming lymphocytes

Abstract. Lymphocytes forming spontaneous rosettes with sheep red blood cells (RFL) have been studied in normal individuals and patients with malignant lymphomas. Rosette formation was temperature dependent and was inhibited by sodium azide or trypsin treatment of lymphocytes, but not by preincubation with an anti-human immunoglobulin IgG preparation. In normals 45–80% of peripheral blood lymphocytes formed rosettes, whereas patients with chronic lymphocytic leukemia and a patient with leukemic IgM producing malignant lymphoma had an extremely reduced percentage of RFL (3–9%). In patients with Hodgkin's disease, lymphocytic lymphosarcoma, reticulum-cell sarcoma, giant follicular lymphoma, Ig producing lymphoma, and multiple myeloma, the proportion of RFL was either diminished or within the normal range (20–63%).

Human peripheral blood lymphocytes represent a heterogeneous population and may be subdivided into different cell types by certain membrane markers. It has been suggested that lymphocytes bearing detectable surface immunoglobulin (Ig) molecules [17, 18, 23, 29] and a receptor for C3 [19, 26, 29, 31] and aggregated IgG [4, 11] are bone marrow-dependent (B) cells. Whereas in unimmunized mice both B and thymus-dependent (T) cells are rosette forming cells [5, 13], human lymphocytes forming spontaneous rosettes with sheep red blood cells (SRBC) are considered as T cells [9, 16, 19, 29, 34]. Evidence for the thymic origin of the rosette forming lymphocytes (RFL) results from the observation that almost all human thymocytes have the ability to form rosettes with SRBC [19, 20, 37] and that RFL are mainly located in the thymus-dependent

[1] Supported by the Alfried-and-Clare Pott Stiftung, Essen.

areas of the lymphatic tissue [32]. Furthermore, rosette formation can be blocked by anti-T cell sera [36], and Ig determinants and a receptor for C3 have not been demonstrated on the surface of RFL [9, 19, 22, 23, 29].

We have studied the percentage of RFL in the peripheral blood of normal individuals and of patients with various malignant lymphomas. In some cases the proportion of lymphocytes with surface Ig has also been determined.

Materials and Methods

Peripheral blood lymphocytes of 23 normal blood donors, 12 patients with chronic lymphocytic leukemia (CLL) and 18 patients with other malignant lymphomas were studied.

For isolation of lymphocytes, freshly drawn heparinized venous blood was allowed to sediment spontaneously at 37 °C. The leukocyte rich plasma was layered on a Ficoll-Isopaque gradient which was prepared by a mixture of 24 parts of 9% Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and 10 parts of 33% Isopaque (*Ronpacon® 75%, Cilag Chemie GmbH, Alsbach, Germany*) as described by BOYUM [6]. After centrifugation at 400 *g* for 40 min at room temperature, the cells were aspirated with a Pasteur pipette and washed twice with Dulbecco's phosphate buffered saline (PBS, pH 7.4, Difco Lab., Detroit, Mich.). Purity of the cell suspension was measured by differential counts of stained smears (95–98% mononuclear cells were present). Cell viability was determined by dye exclusion test using erythrosin B (viability obtained was 95–99%).

For rosette formation, 0.5×10^6 lymphocytes were incubated at 37 °C for 5 min with 5×10^7 washed SRBC in PBS containing 10% heat inactivated (56 °C for 30 min) human AB serum (Grand Island Biol. Co., Grand Island, N.Y.) absorbed with SRBC. The cell mixture was then centrifuged at 100 *g* for 5 min and kept at 4 °C for 30 min. The pellet was gently resuspended by shaking and at least 200 lymphocytes were counted in a hemocytometer. Lymphocytes binding 3 or more SRBC were considered positive as RFL.

In some experiments this procedure was modified by changing temperature and time of incubation. Rosette formation was also determined in the presence of sodium azide (final concentration 0.03%), after pretreatment of the lymphocytes with trypsin (final concentration 0.25%) for 30 min at room temperature and after incubation of the cells with a polyvalent goat anti-human immunoglobulins IgG preparation (anti-human Ig) for 30 min at 4 °C. Fresh SRBC from the same animal were used for all experiments.

For demonstration of surface Ig, 5×10^6 lymphocytes were incubated with a fluorescein-conjugated polyvalent goat anti-human immunoglobulins IgG preparation at 4 °C for 30 min with occasional gentle shaking. The IgG fraction of the goat antiserum to human Ig was prepared by DEAE-cellulose chromatography and was specific for μ, γ, α, $\varkappa$ and λ determinants. Fluorescein conjugation was performed according to the method described by RABELLINO *et al.* [28]. The cells were then

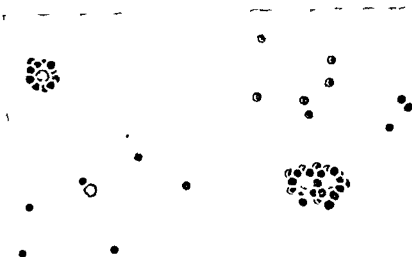


Fig 1 Normal human blood lymphocytes form ng spontaneous rosettes with SRBC

Table 1 Effect of temperature and t me of incubation on rosette formation by normal human lymphocytes (RFL)

Incubation	RFL, %	
	30 m n	24 h
At 4 C	45	38
	53	60
	50	45
	72	70
	80	75
At 37 C	37	4
	20	16
	30	20
	7	2
	18	0

washed 3 t mes w th PBS and were resuspended in 0 5 ml PBS At least 100 cells were exam ned f r membrane fluorescen e at room temperature w: h a Le tz orthoplan m croscope using epi llum nation

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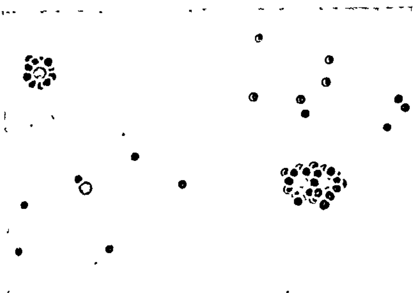


Fig 1 Normal human blood lymphocytes forming spontaneous rosettes with SRBC.

Table I Effect of temperature and time of incubation on rosette formation by normal human lymphocytes (RFL)

Incubation	RFL, %	
	30 min	24 h
At 4 C	45	33
	55	60
	40	45
	72	70
	80	75
At 37 C	37	4
	20	16
	30	20
	7	2
	18	0

washed 3 times with PBS and were resuspended in 0.5 ml PBS. At least 100 cells were examined for membrane fluorescence at room temperature with a Leitz ortho-Plan microscope using epi illumination.

Table II. Percentage of rosette forming normal human lymphocytes at 4°C 30 min and 24 h after pretreatment with trypsin (0.25%) for 30 min at room temperature

Untreated lymphocytes, %	Trypsin treated lymphocytes, %	
	30 min	24 h
58	25	11
62	22	8
53	12	20
69	12	7
56	13	16
46	4	10
64	3	9

Table III. Percentage of rosette-forming normal human lymphocytes during incubation with sodium azide (0.03%) and after preincubation with anti human Ig for 30 min at 4°C

Untreated lymphocytes, %	Treated lymphocytes, %	
	sodium azide	*anti human Ig*
72	–	75
73	68	70
61	47	60
64	38	76
73	22	67
72	42	78

Results

In normal individuals, 45–80% of the peripheral blood lymphocytes formed spontaneous rosettes with SRBC (table I and IV). Typical RFL are shown in figure 1. Table I demonstrates that the percentage of RFL obtained after 24 h of incubation at 4 °C was not significantly different from that after 30 min of incubation. However, the number of RFL was reduced after incubation at 37 °C for 30 min as compared to the control experiments from the same donors at 4 °C and tended to further decrease during incubation for 24 h.

As shown in table II, pretreatment of the lymphocytes with trypsin for 30 min at room temperature inhibited rosette formation as compared to untreated lymphocytes from the same donors, although the cells main-

Table IV. Percentage of rosette forming lymphocytes (RFL) and of lymphocytes with surface bound immunoglobulin (Ig) molecules in the peripheral blood of normal individuals and of patients with chronic lymphocytic leukemia (CLL)

Case No	Diagnosis	RFL, %	Ig-positive lymphocytes %	Blood lymphocytes/μl	Therapy[1]
1	CLL	8	80	5 000	chl, predn
2	CLL	7	88	18,500	–
3	CLL	4	85	20 700	–
4	CLL	4	90	21,300	–
5	CLL	9	87	31,300	–
6	CLL	8	88	31 400	–
7	CLL	4	87	37 400	chl predn
8	CLL	7	78	42,000	–
9	CLL	7	86	47 000	
10	CLL	3	92	51 100	chl
11	CLL	6	92	52,800	–
12	CLL	5	87	53,500	
Normals (n = 23)					
Mean		61	26		
SEM		2	3		
Range		45–80	15–40		

[1] chl = Chlorambucil predn = prednisone

tained good viability as shown by dye exclusion test. During incubation at 4 C for 24 h the lymphocytes did not recover their rosette forming ability. Rosette formation was considerably reduced in the presence of sodium azide (table III). In contrast the percentage of RFL was not significantly altered by preincubation of the cells with polyvalent goat anti human Ig.

Table IV shows that in patients with CLL the percentage of RFL was extremely reduced (3–9%) as compared to normals (45–80%). The majority of CLL cells stained for surface Ig (78–92%) whereas only 15–40% of normal lymphocytes showed membrane immunofluorescence which was characterized by a speckled distribution.

The results obtained with peripheral blood lymphocytes from patients with various malignant lymphomas are presented in table V. Patients with Hodgkin's disease lymphocytic lymphosarcoma reticulum-cell sarcoma', giant follicular lymphoma and multiple myeloma had either normal or

Table V. Percentage of rosette forming lymphocytes (RFL) and of lymphocytes with surface-bound immunoglobulin (Ig) molecules in the peripheral blood of patients with various malignant lymphomas

Case No	Diagnosis	RFL, %	Ig-positive lymphocytes, %	Blood lymphocytes/μl	Therapy[1]
1	Hodgkin's disease (III-B)	63	28	400	c, v, p
2	Hodgkin's disease (IVA)	47	–	450	–
3	Hodgkin's disease (IVB)	42	47	750	p, predn
4	Hodgkin's disease (II A)	42	–	1,200	–
5	Hodgkin's disease (IVB)	45	–	1,700	p, predn
6	Hodgkin's disease (II A)	40	–	1,900	–
7	Hodgkin's disease (II A)	34	48	2,000	–
8	Lymphocytic lymphosarcoma	20	–	550	c, predn
9	Lymphocytic lymphosarcoma	34	–	900	c, v, predn
10	Lymphocytic lymphosarcoma	45	19	1,100	c, v, p, predn
11	'Reticulum-cell sarcoma'	63	–	780	c, predn
12	'Reticulum-cell sarcoma'	60	7	1,000	–
13	Giant follicular lymphoma (generalized)	42	52	1,900	–
14	IgM- and IgA producing malignant lymphoma	34	–	980	–
15	Leukemic IgM producing malignant lymphoma	6	94	43,200	–
16	Multiple myeloma (IgA)	45	–	500	m
17	Multiple myeloma (IgG)	45	35	640	c
18	Multiple myeloma (IgG)	50	24	3,100	–

[1] c = Cyclophosphamide, v = vincristine, p = procarbazine, predn = prednisone, m = melphalan

slightly reduced proportions of RFL. Three of the patients (No 10, 11, 13) had leukemic phases earlier in the course of their disease with a high percentage of Ig-positive cells. At the time of study, only single abnormal blood lymphocytes were detected by light microscopy. These cells stained for surface Ig. One patient (No 15) who suffered from a leukemic IgM-producing malignant lymphoma exhibited an extremely decreased percentage of RFL and a correspondingly increased percentage of lymphocytes with surface Ig. After therapy, the total lymphocyte count and the proportion of Ig-positive cells were within the normal range.

Discussion

Our results confirm the observation that there exist two distinct populations of human lymphocytes – one bearing surface Ig determinants, the other forming spontaneous rosettes with SRBC. The percentages of both lymphocyte populations did not account for exactly 100% in all instances. This finding seems to indicate that there may be a third unclassifiable type of cells. However, in a combined study of rosette forming complement receptor and surface Ig bearing cells, Ross *et al* [29] have demonstrated that all normal blood lymphocytes can be identified as either T or B cells by these criteria.

Spontaneous rosette formation is clearly different from the rosette phenomenon observed with human erythrocytes sensitized with anti-D serum [9] and between Rh positive erythrocytes and blood lymphocytes *from Rh isoimmunized individuals [14]. It seems not to be immunological* in nature, since it is unlikely that such high proportions of lymphocytes have anti SRBC antibodies on their membranes. Indeed, rosette formation is not correlated with the anti SRBC antibody titers which are present in the serum of many donors [8, 20].

The precise nature of the receptor for SRBC on the lymphocyte membrane still is obscure. However, several inferences can be made from the results of our experiments and those of others. Adherence of SRBC to the lymphocyte membrane is temperature dependent [16, 20, 22, 29] and seems to require intact energy metabolism, since sodium azide reduces the percentage of RFL [10, 16]. Pretreatment of the lymphocytes with trypsin abolishes their rosette forming ability [16, 19], indicating that surface structures of protein nature may be involved in the binding of SRBC. Surface Ig receptors appear not to be responsible for rosette formation under the experimental conditions described, since preincubation of the lymphocytes with anti human Ig does not reduce the percentage of RFL. Similar results have been obtained by other investigators using different antibody preparations [7, 10, 16, 19, 22]. However, rosette formation is inhibited by anti human lymphocyte serum [7, 9, 16].

The proportions of RFL found in the peripheral blood of normal individuals correspond to those reported by JONDAL *et al* [19] and ROSS *et al* [29]. In the early studies, a smaller population of normal lymphocytes formed rosettes with SRBC [8, 10, 16, 20, 23, 37]. These differences are most probably due to methodological factors.

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18	Multiple myeloma (IgG)	50	24	3,100	–

[1] c = Cyclophosphamide, v = vincristine, p = procarbazine, predn = prednisone, m = melphalan

slightly reduced proportions of RFL. Three of the patients (No 10, 11, 13) had leukemic phases earlier in the course of their disease with a high percentage of Ig-positive cells. At the time of study, only single abnormal blood lymphocytes were detected by light microscopy. These cells stained for surface Ig. One patient (No 15) who suffered from a leukemic IgM-producing malignant lymphoma exhibited an extremely decreased percentage of RFL and a correspondingly increased percentage of lymphocytes with surface Ig. After therapy, the total lymphocyte count and the proportion of Ig-positive cells were within the normal range.

malignant lymphomas Thus, in these cases detailed studies of the lymphatic tissue itself using a combination of histological and immunological methods are required to establish the T or B cell origin of these lymphatic neoplasias

Note added in proof Recently it has been demonstrated that single human lymphocytes with surface characteristics of B cells may show spontaneous rosette formation with SRBC and thus seem to possess both T and B cell markers (5a)

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Parts of this investigation have been performed as a thesis (A Buka)

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duced in comparison to normals. Similar results have previously been reported in single patients [15, 16, 19, 23, 29]. Recently, AISENBERG *et al.* [2] have found that the proportion of CLL lymphocytes reacting with an anti-human thymocyte serum was diminished. Thus, it may be assumed that there exists a residual population of T cells in the peripheral blood of CLL patients. WYBRAN *et al.* [38] have demonstrated that these cells respond normally to phytomitogens *in vitro*. The majority of lymphocytes from most patients with CLL reveal membrane-bound Ig molecules [1, 18, 23, 27, 35] and a receptor for C3 [26, 31] and aggregated IgG [4, 12] indicating that these leukemic lymphocytes are of B cell origin. However, there have been reports on CLL lymphocytes lacking surface Ig [24, 25, 29] and a complement receptor [21]. Using a combination of all membrane markers it will certainly be possible to classify many of these cases as either T or B cell leukemias [4, 12, 29]. A clear-cut diminution in RFL and a corresponding increase in Ig positive lymphocytes was found in a patient with an unclassifiable leukemic malignant lymphoma producing monoclonal IgM. Thus, the T/B lymphocyte ratio was similar to that observed in the CLL patients indicating that this lymphoma was of B cell type.

The percentage of RFL was slightly diminished or within the normal range in patients with Hodgkin's disease. FRÖLAND [16] reported a normal proportion of this lymphocyte population, whereas AIUTI and WIGZELL [3] observed a reduced number of blood lymphocytes from patients with Hodgkin's disease responding to anti-T cell sera in a cytotoxic assay. Normal or reduced percentages of RFL were also found in patients with lymphocytic lymphosarcoma, 'reticulum-cell sarcoma', giant follicular lymphoma and multiple myeloma. In 3 cases single abnormal cells which stained for surface Ig were circulating in the peripheral blood. Some of our patients were under cytostatic treatment at the time of study and/or had previously undergone radiotherapy. Since irradiation has been shown to cause a decrease in T cells and an increase in B cells [34], the possibility has to be considered that in these cases therapy has at least in part contributed to the changes in the ratio of both lymphocyte populations.

Evidence from our findings suggests that in contrast to leukemic lymphomas with high blood lymphocyte counts the determination of blood lymphocytes with T or B cell characteristics does not contribute to the differentiation between the various forms of nonleukemic malignant lymphomas. Also, it has recently been emphasized by STEIN *et al.* [33] that histological criteria alone are insufficient for the exact classification of

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PHA Response of Blood and Lymph Node Lymphocytes *in vitro* in Malignant Lymphomas

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Abstract. *In vitro* blastic phytohaemagglutinin (PHA) transformation of blood and lymph node lymphocytes from cases of lymphadenitis, malignant lymphomas, and chronic lymphocytic leukaemia (CLL) has been studied. In lymphosarcoma and CLL the percentage of PHA transformed lymph node lymphocytes was low, thus suggesting that such lymphocytes are of the B type. In Hodgkin's disease, blood and lymph node lymphocytes gave PHA transformation nearly as high as in controls: this suggests that T lymphocytes are not necessarily involved in Hodgkin's disease.

Key Words
Lymphadenitis
Lymphocyte transformation
Lymphocyte types (B, T)
Malignant lymphomas
Phytohaemagglutinin

In vitro blastic PHA transformation of peripheral blood lymphocytes in malignant lymphoproliferative disorders has been investigated extensively with conflicting results. In blood cultures from cases of Hodgkin's disease, the percentage of PHA-transformed cells has been reported to be either decreased [16, 17, 21], normal [8, 26], or varying from case to case [6, 13, 31]. In cases of lymphosarcoma or reticulum cell sarcoma, the PHA response of blood lymphocytes has been found to be as high as in normal subjects [31, 33] or decreased [5, 15, 23]. On the other hand, in CLL the PHA response of peripheral blood lymphocytes has constantly been reported to be very low [4, 5, 8, 14, 23, 27, 31, 33]. Therefore, it seems that, with the exception of CLL, the peripheral blood cytology does not reflect exactly the pathological status of lymphoid cells at the sites primarily involved in lymphoproliferative disorders.

The purpose of this investigation was to study the *in vitro* PHA transformation of lymphoid cells obtained from lymph nodes in malignant lymphomas and CLL. A few studies along this line have already been re-

ported [1, 28, 36] including one from this laboratory [30]. In these studies performed on a small number of cases, conflicting results have been obtained regarding lymphosarcomas [1, 28, 36], and normal behaviour has been observed in the 3 cases of Hodgkin's disease so far investigated [36]. More extensive research is presented in this paper, and the results are evaluated in the light of recent data about T (thymus-derived) and B (bone marrow-derived) lymphocytes.

Materials and Methods

Lymph nodes removed for diagnostic purposes from 10 cases of lymphadenitis (used as controls), 29 untreated malignant lymphomas (18 Hodgkin's diseases and 11 lymphosarcomas) and 14 untreated CLLs have been studied, as well as blood samples of all patients. Peripheral blood samples were obtained the day before the biopsy, and *in vitro* cultures were performed as described elsewhere [34] (mechanical defibrination, gelatin separation of white cells, inoculum of 2 · 10^6/ml of mononuclear cells in 10-ml cultures of TC 199 Wellcome with antibiotics and 10% autologous serum). In all cases, the cultures were performed both with and without addition of PHA (Wellcome, 0.02 ml/ml).

Lymph nodes were finely minced in TC 199; after a brief sedimentation of the tissue fragments, the cell rich supernatant was diluted in TC 199 with 10% autologous serum to obtain a final cell concentration of 2 · 10^6/ml, and cultures both with and without PHA were performed as above.

Histological diagnosis on the lymph nodes were made according to the criteria adopted in this laboratory for lymphomas [29]. Hodgkin's disease was histologically classified according to LUKES and BUTLER [19] in lymphocytic and/or histiocytic, nodular sclerosis, mixed, diffuse fibrosis, and reticular types. The lymphosarcomas were divided into well and poorly differentiated types according to RAPPAPORT [25].

Results

In normal subjects (table I), the percentage of PHA transformed cells was similar in peripheral blood and in lymph node cultures (72.4 ± 4.8 and 66.4 ± 7.7%, respectively; mean ± standard deviation).

In Hodgkin's disease (table II), the PHA lymphocyte transformation was slightly decreased both in blood (55.6 ± 12.7%) and lymph node cultures (49.5 ± 10.5%), although individual variations were conspicuous. In lymph node cultures, the highest percentages were obtained from lymphocytic or lymphocytic-histiocytic types, and the lowest ones from diffuse fibrosis types. No great discrepancies were observed between blood and lymph node cultures.

Table I *In vitro* PHA transformation of blood and lymph node lymphocytes in lymph adenitis

Case No	PHA blastic transformation, %	
	blood	lymph node
1	65	57
2	72	72
3	76	78
4	78	75
5	72	64
6	66	64
7	69	57
8	73	58
9	73	66
10	80	73
Mean ± SD	72.4 ± 4.8	66.4 ± 7.7

Table II *In vitro* PHA transformation of blood and lymph node lymphocytes in Hodgkin's disease

Case No	PHA blastic transformation, %		Type
	blood	lymph node	
1	79	66	lymphocytic
2	54	67	lymphocytic
3	59	50	lymphocytic
4	69	59	lymphocytic
5	61	41	lymphocytic histiocytic
6	59	51	lymphocytic histiocytic
7	59	48	nodular sclerosis
8	51	40	nodular sclerosis
9	45	59	nodular sclerosis
10	56	48	nodular sclerosis
11	74	62	nodular sclerosis
12	50	45	nodular sclerosis
13	36	45	mixed
14	61	46	mixed
15	42	36	mixed
16	70	59	mixed
17	43	30	diffuse fibrosis
18	34	40	diffuse fibrosis
Mean ± SD	55.6 ± 12.7	49.5 ± 10.5	

Table III In vitro PHA transformation of blood and lymph node lymphocytes in lymphosarcoma

Case No	PHA blastic transformation, %		Type
	blood	lymph node	
1	50	24	well differentiated
2	55	8	well differentiated
3	63	21	well differentiated
4	49	23	well differentiated
5	61	7	well differentiated
6	62	12	well differentiated
7	70	9	well differentiated
8	73	36	poorly differentiated
9	74	35	poorly differentiated
10	66	30	poorly differentiated
11	69	31	poorly differentiated
Mean ± SD	62.9 ± 8.6	21.4 ± 10.9	

Table II In vitro PHA transformation of blood and lymph node lymphocytes in chronic lymphocytic leukaemia

Case No	PHA blastic transformation, %	
	blood	lymph node
1	5	7
2	0	6
3	1	3
4	17	9
5	0	1
6	5	4
7	1	4
8	5	8
9	3	6
10	3	3
11	1	2
12	1	8
13	2	1
14	15	8
Mean ± SD	4.2 ± 5.3	5.0 ± 2.8

In cases of lymphosarcoma (table III), the mean PHA blastic transformation of blood lymphocytes was always nearly as high as in normal subjects (62.9±8.6%). On the other hand, it was consistently decreased in lymph node cultures (21.4±10.9%), lower values were observed in well differentiated lymphosarcomas than in poorly differentiated ones.

In CLL (table IV) very few PHA blast cells were consistently obtained from either blood or lymph node lymphocytes (4.2±5.3 and 5.0±2.8%, respectively).

Blood and lymph node cultures untreated with PHA constantly showed a blast cell percentage below 2 and 6%, respectively; this applied to both normal and lymphomatous subjects.

Discussion

It has been suggested that the PHA responsivity is peculiar to T lymphocytes [7, 32]. Thus, blood and lymph node lymphoid cells showing high PHA responsivity could be referred to as T lymphocytes. On the other hand, a low PHA responsivity would suggest that the cells belong to B lymphocytes [3].

Therefore the low PHA blast cell percentage in blood and lymph node cultures from CLL patients could indicate that the leukaemic cells belong to the PHA-unresponsive B lymphocytes. This hypothesis, formulated by MILLER [20] on the basis of immunological and epidemiological data, is also supported by the findings that membrane-bound immunoglobulins peculiar of B lymphocytes in the mouse [24] and in man [9], have been observed in CLL cells [18, 22, 35].

Similarly, the low percentage of PHA responsive cells obtained from lymph node lymphocytes in all our cases of lymphosarcoma suggests that the neoplastic cells in this lymphoproliferative disorder are also B lymphocytes, as MILLER [20] previously postulated. This hypothesis would agree with the findings of AISENBERG and BLOCH [2]. In 3 of 4 cases of lymphocytic lymphoma they demonstrated high density of immunoglobulins on lymph node lymphoid cell surface.

The blood lymphocytes of lymphosarcomas in our series behaved normally with PHA *in vitro*. This disagrees with the low PHA blastic transformation reported by others [5, 23]. In some reports no mention was made of possible haematic dissemination of lymphosarcoma cells; when the latter was recognized, it was found that such cells also carry membrane bound immunoglobulins [10].

The high PHA blastic response of lymph node lymphocytes in Hodgkin's disease in our series (although variable from case to case and dependent on the histological pattern of lymph nodes) shows that, as a rule, T lymphocytes are conspicuously present in involved lymph nodes. This finding cannot exclude the interpretation of Hodgkin's disease as a disorder of the thymus-dependent system [11, 20] but it suggests that the T lymphocytes of the lymph nodes are unaffected at least as far as PHA responsivity is concerned.

Individual differences have been reported in the literature on the percentage of PHA responsive peripheral blood lymphocytes in Hodgkin's disease, whereas only a slight decrease of PHA responsivity has been observed in our series. Some investigators have related such differences to the clinical stage of Hodgkin's disease [15, 21, 33]. However, the role of therapy and of plasmatic factor(s) must also be considered [12, 13, 15]. In any case, the lowering of PHA lymphocyte transformation in Hodgkin's disease is not as marked as to indicate an extensive pathological change of lymphoid population.

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A Study of Lymphocytic β-Glucuronidase in Various Benign and Malignant Lymphatic Processes

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Abstract Lymphocyte β glucuronidase activity of 50 normal controls and of 101 patients with benign or malignant lymphatic processes, using Lorbacher's technique, was studied A constant and statistically significant decrease has been found in chronic lymphocytic leukaemia This and other results are briefly discussed The study of lymphocytic β glucuronidase can provide useful information in the diagnosis of both peripheral and central lymphocytoses

Key Words
Cytochemistry
β Glucuronidase
Infectious mononucleosis
Lymphocytes
Lymphocytic leukaemia
Lymphoproliferative disorders

In 1947, Fishman and Anlyan [1] succeeded in demonstrating for the first time β-glucuronidase activity in blood cells by biochemical techniques The study of this hydrolase by cytochemical methods did not acquire widespread acceptance until recently, due in part to technical difficulties, to the enzyme's great lability and to its limited activity in the haemopoietic cells The introduction of Lorbacher's technique [2] using naphthol-As Bi-glucuronide as a substrate, opened the way to the cytochemical study of this hydrolytic enzyme

The reports of several authors [2–4], aroused our interest to investigate the β-glucuronidase activity in lymphatic processes, both benign and malignant The scanty existing literature indicates a constant marked decrease of enzymatic activity in chronic lymphatic leukaemia Such an alteration is of great practical interest, as it permits identification through simple optical microscopy of pathological lymphocytes, which from strictly morphological aspects hardly or not at all differ from normal ones Furthermore, as in the case of granulocytic alkaline phosphatase in the

myeloproliferative disorders, β-glucuronidase can serve as a characteristic sign in the differential diagnosis of the lymphocytoses.

Materials and Methods

In the present study lymphocytic β glucuronidase activity was determined in 50 normal controls, varying in age from 3 months to 82 years, and in 101 benign and malignant lymphatic processes. For the cytochemical demonstration of β glucuronidase Lorbacher's technique [2] with the following slight modifications was used.

Stock solution of naphthol AS BI glucuronide substrate. Dissolve 28 mg of acid free naphthol AS BI glucuronide completely in 1.2 mg of 0.05 M sodium bicarbonate (420 mg of sodium bicarbonate in 1000 ml of distilled water). Then add 0.2 M acetate buffer till 100 ml.

Buffer. Solution A: 0.2 M acetic acid (12 g/l of distilled water), solution B: 16.4 g of anhydrous sodium acetate in 1 l of distilled water. Mix 14.8 ml of solution A with 35.2 ml of solution B. This work solution may be stored several weeks at room temperature.

Pararosaniline solution. Dissolve 1 g of hydrochloric pararosaniline in 20 ml of distilled water, add 5 ml of concentrated hydrochloric acid, shake gently and heat. Once the solution cools, filter it and store in the refrigerator.

Sodium nitrite solution. Sodium nitrite at 4% in distilled water. It is advisable to prepare it the same day.

Technical instructions for incubation. Use blood smears air-dried at room temperature for 1–6 h. Fix for 60 sec with methanol formol (3:7) at 4 °C, rinse with distilled water and dry for 30 min at room temperature. Freeze the smears for 60 min. Incubate at 37 °C during 1 h with the following mixture.

Incubation mixture. Mix 0.3 ml of pararosaniline solution with an equal amount of sodium nitrite solution. Shake for 1 min. Add the stock solution (10 ml) and adjust the pH to 5.2 with 1 N bicarbonate using a pH meter. Add distilled water to reach a volume of 20 ml. Filter with a Whatmann No. 1 filter, rinse with distilled water and dry at room temperature. Contrast the smears with Harris' haematoxylin for 20 min.

Evaluation. In this study only a percentual count of β-glucuronidase positive lymphocytes was performed. Some authors, e.g. Yam and Mitus [5], measure the activity in a semi-quantitative way dividing the lymphocytes into four types according to the number and size of granules resulting from the enzymatic activity. As a rule, the positive reaction is seen in granular form although, on rare occasions, a faint diffuse cytoplasmic distribution may appear. The granules whose colour ranges from brilliant red to orange may be solitary or multiple and are scattered uniformly throughout the cytoplasm.

To be able to evaluate activity accurately, it is necessary to know the precise cytological distribution of this enzyme. In fact it can be found in the majority of bone marrow and blood cells. The reaction with Lorbacher's technique is strongly positive in macrophages, monocytes and some lymphocytes. In phytohaemagglutinine-transformed lymphocytes, β glucuronidase activity is even more intense. It

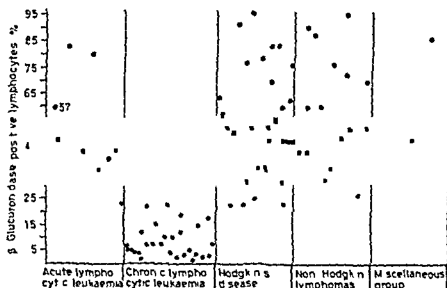


Fig 1 Lymphocytic β glucuronidase in various malignant lymphatic processes

is moderate in the granulopoietic and thrombocytic series and very scanty or absent in erythrocytes and their precursors Using the ferric hydroquinoline technique FORTEZA BOVER [8] failed to obtain a lymphocyte positive reaction JANIGAN and PEARSE [9] are of the opinion that it may be due to a lesser sensitivity and specificity of said technique

Ultrastructural studies show that β glucuronidase is located in lysosomes and also in some parts of the Golgi complex [7]

Results

The normal controls did not present noteworthy differences in their enzymatic activity as far as age and sex are concerned The mean value of β glucuronidase positive lymphocytes was 44% with a standard deviation of ±13

Malignant Lymphoproliferative Disorders (fig 1)

Chronic lymphocytic leukaemia Different authors [2–5, 10] have repeatedly observed a β glucuronidase decrease in chronic lymphocytic leukaemia In this study of 28 patients (table I) low enzyme activity was also observed 24 patients with no other concomitant pathology were found to have a mean value of 5 7 with a standard deviation of ±3 5 which is sta

Table I Lymphocytic β-glucuron dase in 28 patients with chron c lymphatic leukaem a

Patient	WBC/mm³	Lympho-cytes, %	β-Glucuronidase posit ve lymphocytes, %
S C.	56 000	89	6
S R	24,500	85	5
B M	1 000 000	99	4
M C	60 000	85	4
C R	24 000	90	4
A C	120 000	90	1
S S	8 000	65	12
G L.	70 000	97	21 (infect on)
A CH	14,500	84	7
A M.	41,000	88	7
C N	20 000	84	15 (d abetes)
M B	48 000	82	7
J R.	5,200	25	22 (rem ssion)
A. F	35 000	90	10
G T	36,000	90	3
A F	13 000	85	10
E M.	15 000	81	2
G N	50 000	90	12
L. L.	46,000	87	19
E. S.	50 000	94	3
A L.	70 000	91	5
S B	97 000	96	1
F N	20,000	91	3
M C	20 000	87	15
J M	114 000	96	3
M L.	6 000	56	18 (remission)
A B	40 000	64	4
J E.	15 000	76	6

tistically very significant ($p = 0.000001$) In 2 patients (J R and M L.) after the clinical and haematological remission was achieved a rise to subnormal limits was noted with a mean value of 20 Two other patients showing similar values had associated diseases (one diabetes mellitus, the other urinary infection) From observation of these cases it can be concluded that the decrease of β-glucuronidase activity in established chronic lymphocytic leukaemia is the rule A lymphocytic β glucuronidase increase in agreement with other authors is another indicator of remission although it may also be due to concomitant pathology

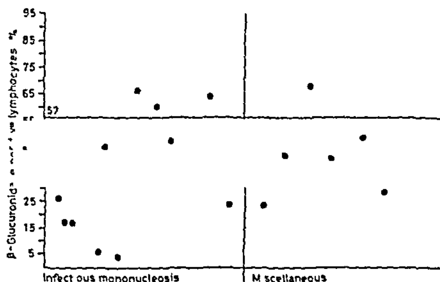


Fig. 2. Lymphocytic β glucuronidase in various benign lymphatic reactions.

Acute lymphoblastic leukaemia. In this disorder, in contrast to the chronic one, the lymphocytic β-glucuronidase values did not differ greatly from the normal ones (fig. 1). The blast cells are very seldom positive. The forementioned technique does not serve as a tool in the classification of acute leukaemia.

Hodgkin's disease. In 29 patients the β glucuronidase values were within or above normal limits, which agree with earlier reports [5, 10]. Significant differences have not been noted among histological types nor among different clinical stages of the disease.

Non Hodgkin lymphomas. In 15 patients suffering from other types of lymphomas, enzymatic activity was found to overlap that found in Hodgkin's disease, regardless of cell type (lymphocytic or histiocytic) or histological structure (nodular or diffuse). This is in contrast with the results reported by YAM and MITUS [5] who obtained low values in 4 out of 7 lymphosarcomas. However, the scanty number of cases studied does not permit us to draw any definite conclusions.

Miscellaneous group. Two patients with Waldenstrom's macroglobulinaemia, one with normal and one with high values, have been included in this group. The results observed, if confirmed in future observations, may be of interest in the differential diagnosis between chronic lymphocytic leukaemia and macroglobulinaemia, since both diseases show lymphatic infiltration of bone marrow. Decreased activity of β glucuronidase in pro

liferative cells would suggest chronic lymphocytic leukaemia and increased or normal levels, on the other hand, would point to a probable diagnosis of Waldenström's macroglobulinaemia

Benign Lymphatic Processes (fig 2)

Infectious mononucleosis This group includes 11 patients with clinical and cytological pictures characteristic of mononucleosis YAM and MITUS [5] found β glucuronidase activity within the normal range In half of our cases the values were markedly low or subnormal at the beginning of the clinical period A new analysis a few weeks later showed a return to normal, a finding which does not take place in chronic lymphocytic leukaemia However, this differential diagnosis does not present itself from the cytological point of view

Miscellaneous group This group was composed of a post-exanthematic lymphocytosis of unknown aetiology, another due to poliomyelitis, one to toxoplasmosis and three cases of whooping cough In all of them β glucuronidase values did not undergo noteworthy modifications

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Anomalies immunitaires au cours des splénomégalies myéloïdes avec myélosclérose

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Key Words
Antibodies in splenomegaly
Myelofibrosis
Myeloid splenomegaly
Splenomegaly

Abstract. In a study of 26 cases of myeloid splenomegaly with myelofibrosis, the authors find a large incidence of immunization anomalies, principally positive latex reaction in 8 cases, monoclonal proteins in 3 cases, anti-erythrocyte antibodies in 6 cases. Anti muscle antibodies, quantitative changes of the immunoglobulins and the presence of cryoglobulin are also observed.

In a control group of 28 patients of identical age distribution, afflicted with various types of hemopathies, immunization anomalies are encountered far more rarely. The significance of immunization anomalies in the course of myeloid splenomegaly with myelosclerosis is discussed especially in its relationship with the development of medullary fibrosis.

La splénomégalie myéloïde avec myélosclérose est un syndrome relevant rarement d'une étiologie connue et apparaissant le plus souvent soit comme une maladie primitive, soit comme le terme évolutif de la polyglobulie vraie de Vaquez. Elle est généralement considérée comme un syndrome myélo-prolifératif au cours duquel se développe, par un mécanisme inconnu, une réticulofibrose médullaire progressivement croissante. La maladie respecte le système lymphoïde et ne semble pas, à priori, prédisposer à l'apparition de désordres immunitaires. Quelques observations publiées font état de rares associations avec des anémies hémolytiques à auto-anticorps et, dans une très courte série antérieurement rapportée [5], nous avions constaté une fréquence inattendue d'anomalies immunitaires. Le présent travail rapporte une expérience plus étendue et comparative qui confirme nos conclusions préliminaires.

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Matériel et méthodes

Deux groupes de malades ont été inclus dans cette étude 26 sujets atteints de splénomégalie myéloïde primitive et 28 sujets porteurs d hémopathies diverses cons tituant un groupe témoin

Dans le premier groupe, 21 malades ont une splénomégalie myeloide primitive chez 5 sujets elle est secondaire à une maladie de Vaquez traitée Dans tous les cas, le diagnostic est affirmé par la constatation d une splénomégalie, de modifications hématologiques périphériques habituelles en particulier d une discrète myélo-éry throblastémie, et par la biopsie médullaire (Prof agr F POTET, Laboratoire central d Anatomie pathologique, Hôpital Beaujon Clichy) Onze hommes et 15 femmes ont été étudiés L'âge des malades s'échelonne entre 40 et 75 ans 5 sujets ont entre 40 et 49 ans, 3, entre 50 et 59 ans, 10 entre 60 et 69 ans, et 8 plus de 70 ans La durée de l évolution clinique connue avant que ne soit pratiqué le bilan immunologique sérique est de moins de 2 ans chez 13 malades de 2 à 5 ans chez 9, et de 5 à 17 ans chez 4

La splénomégalie est modérée (débord de moins de 4 travers de doigts sous le rebord costal) chez 9 sujets, elle est moyenne (pôle inférieur ne dépassant pas la ligne horizontale de l'ombilic chez 12 sujets, elle est très volumineuse chez 5 malades

Les lésions médullaires ont été classées par ordre de gravité croissante en 3 catégories chez 8 malades, on note un épaississement de la trame réticulinique sans fibrose collagène, chez 9 malades un même épaississement associé à une fibrose col lagène laissant persister une partie du tissu hématopoïétique chez 9, une myélosclé rose très importante avec raréfaction très marquée du tissu hématopoïétique

Outre les manifestations d athérosclérose fréquente chez les sujets de plus de 60 ans, les manifestations pathologiques associées à la maladie hématologique ont été les suivantes dans 1 cas, une insuffisance rénale sévère due à une pyélonéphrite chronique dans 1 cas, un cancer du sein chez 1 malade une hypertrophie nodulaire partielle du foie ou maladie de Sherlock chez 1 autre une thrombose portale secon daire et une lithiase cholédocienne, chez 1 malade une syphilis tertiaire

Le bilan immunologique sérique pratiqué a comporté les examens suivants

recherche de cellules LE d anticorps anti nucléaires, anti mitochondries anti muscle lisse et strié anti-estomac et anti facteur intrinsèque anti thyroide et anti épiderme par immunofluorescence (Laboratoire de la Banque du Sang Dr P JAULIN Hôpital Bichat, Paris),

recherche du facteur rhumatoide par la réaction du latex sur lame et en tube et par la réaction de Waaler Rose, immuno-électrophorèse des protéines sériques

recherche d une cryoglobuline (D N SCHLEGEL, Laboratoire central d Hémato logie Hopital Beaujon Clichy)

examen immuno-hématologique comprenant test de Coombs direct à 4 et à 37 °C, élution à 56 °C et tests sur les éluats à 37, 22 et 4 °C, sensibilisation par les enzymes protéolytiques recherche et dosage des anticorps froids agglutinants (Dr M WROCLANS, Laboratoire du Centre de transfusion sanguine, Hôpital Beaujon Clichy)

Chez quelques malades, pour des raisons techniques, certaines de ces épreuves n ont pas été pratiquées

Les mêmes examens biologiques ont été pratiqués pendant la même période de temps chez 28 malades atteints d'autres variétés d'hémopathies, constituant ainsi un groupe témoin : 12 « lymphomes » dont 5 leucémies lymphoïdes chroniques, 6 lymphosarcomes, 1 maladie de Hodgkin, 4 anémies carentielles en vitamine B_{12} ou en folates, 4 cancers viscéraux, 4 purpuras thrombopéniques par destruction périphérique, 2 anémies hémolytiques congénitales, 3 insuffisances médullaires primitives dont 1 anémie sidéroblastique. La répartition de ces malades par tranches d'âge est très voisine de celle des malades atteints de splénomégalie myéloïde avec myélosclérose : 6 sujets de moins de 50 ans, 1 sujet entre 50 et 59 ans, 7 sujets entre 60 et 69 ans, 11 sujets entre 70 et 79 ans, 3 sujets de plus de 80 ans. Au total, sur 28 sujets de ce groupe, 21 avaient plus de 60 ans, sur 26 malades atteints de splénomégalie myéloïde avec myélofibrose 18 avaient plus de 60 ans, les deux groupes sont donc sur ce point comparables.

Résultats

Splénomégalies myéloïdes avec myélofibrose

La recherche des cellules LE a été positive 1 fois sur 19 à une dilution de 1/40. Des anticorps anti nucléaires ont été trouvés 3 fois, à des dilutions de 1/50, 1/50 et 1/10. Une fois la réaction était douteuse avec du sérum non dilué. Chez 3 malades ont été trouvés des anticorps anti-muscle lisse, chez 1 malade un anticorps anti-estomac et anti-facteur intrinsèque sans aucun trouble de l'absorption de la vitamine B_{12} radio-active. Chez aucun des 23 malades où ils ont été recherchés n'ont été trouvés d'anticorps anti thyroïde, anti-muscle strié, anti-épiderme et anti-mitochondries.

La réaction au latex a été trouvée positive sur lame et en tube chez 8 malades sur 18. Chez une de ces malades, la réaction positive à 1/40 n'est pas significative, mais elle demeure positive à plusieurs examens. Chez 7 malades sur 8, la réaction est positive à des dilutions égales ou supérieures à 1/320, chez 4 malades sur 6 à des dilutions égales ou supérieures à 1/1280. Dans un cas elle l'est à 1/200 000. La réaction de Waaler Rose a été trouvée positive 3 fois sur 18 à des titres allant de 1/16 à 1/128.

L'immuno-électrophorèse des protéines sériques est normale chez 12 malades sur 23. Une élévation modérée des IgG est notée 6 fois, une augmentation des IgM, 5 fois. La recherche de cryoglobuline a été positive chez 3 malades sur 24. Chez 3 malades a été décelée une protéine monoclonale : 2 fois IgGκ et 1 fois IgMκ. L'examen immuno-hématologique est négatif chez 20 malades sur 26. Chez 4 malades le test de Coombs direct est positif, l'anticorps est une IgM de spécificité anti I dans tous les

cas. Chez une malade, une grande anémie hémolytique à auto-anticorps froids (agglutinines froides positives à 1/2500) a précédé d'un an l'apparition de la myélofibrose et de la splénomégalie myéloïde. Chez les 3 autres malades, l'hyperhémolyse est modérée. Chez 2 malades, le test d'élution à 4°C met en évidence un auto-anticorps anti-I en l'absence de positivité du test de Coombs.

Au total, une ou plusieurs anomalies immunologiques sériques existent chez 19 malades sur 26. Aucune anomalie n'a été trouvée chez 7 malades sur 26. Si l'on tient pour incertaines les modifications des immunoglobulines décelées par immuno-électrophorèse, en l'absence de dosage systématique des différentes classes par la méthode de Mancini, 31 anomalies majeures sont rencontrées sur 26 malades au total.

En fonction de l'âge des malades, une ou plusieurs anomalies immunologiques sériques existaient chez 3 malades sur 5, d'âge compris entre 40 et 49 ans, chez 2 malades sur 3 ayant entre 50 et 59 ans, chez 7 malades sur 10 ayant entre 60 et 69 ans et chez 4 malades sur 8 ayant plus de 70 ans. Cinq hommes sur 11 et 9 femmes sur 15 étaient porteurs d'anomalies. En fonction de la durée de l'évolution clinique de la splénomégalie myéloïde et/ou de la myélosclérose, une ou plusieurs anomalies ont été notées chez 11 malades sur 13 ayant une évolution inférieure à 2 ans, chez 4 malades sur 8 ayant une évolution comprise entre 2 et 5 ans, chez 3 des 5 malades ayant une évolution supérieure à 5 ans. En fonction de l'intensité des lésions médullaires: parmi les 8 malades dont les lésions sont peu importantes et caractérisées par une augmentation de la trame réticulinique sans fibrose collagène, 4 sont porteurs d'une ou plusieurs anomalies; il en est ainsi chez 7 des 9 malades ayant une fibrose d'intensité moyenne et chez 7 des 9 malades ayant une ostéomyélosclérose très importante.

Une tentative de recherche des corrélations a été menée entre, d'une part le type de l'anomalie décelée, et d'autre part l'âge des malades, la durée de l'évolution clinique et l'intensité de la myélosclérose. En fonction de l'âge, il n'apparaît pas avec évidence de différence significative de type d'anomalie. En fonction de la durée d'évolution clinique, il semble ressortir que les anomalies souvent multiples apparaissent assez tôt dans l'évolution puisque tous les types d'anomalies sont représentées dans le groupe de malades dont la durée d'évolution clinique est inférieure à 2 ans. En fonction du degré de la myélosclérose, il semble que les anticorps anti-nucléaires, anti-tissus, et anti-érythrocytaires d'une part, la possibilité de la réaction au latex de l'autre, soient plus fréquentes lorsque la myélo-

fibrose est importante Au total, ces divers types d anomalies s'observent sans rapport étroit avec l âge, lors des myéloscléroses importantes, évoluant depuis relativement peu de temps, leur présence aurait donc une certaine signification d évolutivité de la maladie

Groupe témoin

Un anticorps anti nucléaire a été décelé 1 fois au cours d une carence en folates chez un homme de 74 ans avec une dilution à 1/25 La recherche d'anticorps anti muscle lisse a été 2 fois positive à 1/25 et à 1/50 chez 2 malades atteints de lymphosarcomes. Aucun autre anticorps anti tissu n'a été décelé Les réactions au latex et de Waaler Rose ont été négatives chez tous les malades du groupe L'immuno-électrophorese a mis en évidence une diminution des immunoglobulines chez plusieurs malades atteints de lymphomes Aucune protéine monoclonale n'a été décelée Une cryoglobuline mixte IgG IgM a été trouvée chez une malade atteinte de la maladie de Biermer Le test de Coombs direct a été trouvé 2 fois positif au cours d une anémie sidéroblastique (IgM anti I) et au cours d'un cancer thyroïdien avec métastases diffuses (IgM anti I avec agglutinines froides, augmentées au taux de 1/2048) Chez 4 malades, un anticorps anti I a été décelé par la méthode de l'élution à 4 °C alors que le test de Coombs direct était négatif 1 maladie de Biermer, 1 purpura thrombopénique aigu, 2 lymphomes Au total, et en ne tenant pas compte de l interprétation quantitative des immuno-électrophorèses, comme pour le groupe des splénomégalies myéloïdes, 9 anomalies immunologiques ont été décelées dans le groupe témoin

Discussion

La mise en évidence d anomalies immunologiques de haute incidence, au cours d une affection généralement considérée comme un syndrome myélo-prolifératif est, à priori, assez surprenante Les anomalies sont diverses, parfois associées et la plus fréquente d entre elles est la positivité de la réaction au latex.

Le premier problème qui se pose est celui de la possibilité d une coïncidence entre la présence de désordres immunitaires séniques et celle de la splénomégalie myéloïde avec myélofibrose Cette maladie s'observe en effet au-delà de l âge moyen de la vie, généralement après 50 ans. Or il est connu que chez le sujet âgé peuvent exister divers types d anomalies

immunologiques en l'absence d'une maladie causale reconnue et particulièrement une positivité de la réaction au latex. Dans les populations d'âge moyen, une telle positivité est trouvée chez 1–3% des sujets [2, 4, 36]. Au-delà de 65 ans, l'incidence est nettement plus importante, encore que variable selon les auteurs: 11% pour Jacqueline *et al.* [22], 16% pour Cammarata *et al.* [9], 28% pour Waller *et al.* [36], 30% pour Litwin et Singer [30] et pour Heimer *et al.* [17], 42% pour Bach *et al.* [3]. Des anticorps anti-nucléaires pourraient être mis en évidence [2, 9] avec une incidence importante (36% selon Cammarata *et al.* [9]. La présence de protéines monoclonales en dehors de myélome ou de macroglobulinémie de Waldenström a également été rapportée [8, 13]. Ces diverses anomalies se rencontrent essentiellement chez le sujet âgé et sont d'autant plus fréquentes que l'âge est plus avancé. Dans la présente étude, des désordres immunitaires sont trouvés, certes avec une grande fréquence chez les sujets de plus de 60 ans mais aussi chez les malades plus jeunes: ainsi la réaction du latex est positive chez 2 des 5 malades situés dans la tranche d'âge de 40 à 49 ans, ce qui ne laisse que peu de risques d'une coïncidence due à l'âge. Par ailleurs, dans le groupe témoin ayant une répartition d'âge analogue, l'incidence des anomalies est nettement inférieure.

L'existence d'une autre affection susceptible d'entraîner la présence de désordres immunitaires mérite également d'être discutée. Aucun de nos malades n'avait un tableau de maladie rhumatoïde et une seule a présenté des manifestations articulaires douloureuses. Une affection hépatique [4, 10, 11, 17, 21], en particulier une hépatite chronique est possible chez ces malades dont certains ont été polytransfusés, même si la recherche de l'antigène Australia est négative. Une telle hypothèse pourrait être discutée chez plusieurs de nos malades. Chez l'un d'eux, en effet, la réaction au latex est devenue positive après une hépatite d'inoculation, alors qu'elle était négative 6 mois auparavant. Par contre, les anomalies immunitaires existaient avant toute atteinte hépatique clinique et biologique chez une malade et en l'absence de celles-ci chez 3 autres malades. En outre, il n'y a pas de corrélation entre la présence de ces désordres et les antécédents de transfusion antérieure: ils sont présents chez des sujets qui n'ont jamais été transfusés. L'existence chez une malade d'une hypertrophie nodulaire partielle du foie ne paraît pas expliquer aisément la présence d'un anticorps anti-estomac. Celle d'un cancer du sein chez une malade aurait pu rendre compte de la myélosclérose; la biopsie médullaire ne montrait pas de métastase mais la positivité des réactions

au latex et de Waaler Rose est peut-être à mettre au compte de désordres immunitaires secondaires au cancer lui même. Aucune relation par contre n'apparaît entre les désordres immunitaires et les traitements médicamenteux reçus par les malades (corticoïdes, ^{32}P, anabolisants, chimiothérapie cytolytique).

La littérature est assez pauvre en documents sur l'état immunologique au cours des splénomégalies myéloïdes avec myélofibrose. L'association à une anémie hémolytique à auto-anticorps a été signalée à plusieurs reprises [6, 19, 23, 29, 32, 33] parfois même, comme chez l'une de nos malades, l'anémie hémolytique précède l'apparition de la myélosclérose [23]. On connaît également la fréquence de la positivité des tests de la maladie de Marchiafava Micheli au cours des myéloscléroses [28]. La présence d'une cryoglobuline, celle d'une immunoglobuline monoclonale [12, 13] ont été occasionnellement rencontrées. L'association d'un myélome multiple avec une splénomégalie myéloïde avec myélofibrose est peut-être à en rapprocher [7, 20, 25, 34, 35]. Quelques cas d'association de polyglobulie vraie avec des myélomes ont été rapportés [7, 14–16, 18, 24, 27, 35], si l'affirmation de la plasmocytose maligne est appuyée par la présence de lésions osseuses destructrices dans plusieurs de ces cas, l'association de polyglobulie et de protéine monoclonale semble possible en l'absence de preuve de l'existence d'un myélome vrai [31]. Dans nos cas d'association de protéine monoclonale et de myélofibrose, il n'y avait pas de prolifération plasmocytaire décelable et aucune lésion osseuse destructrice.

Caplan [10], en 1963, a signalé la positivité de la réaction au latex sur lame dans 5 cas sur 8 de splénomégalie myéloïde avec myélosclérose, mais aussi dans 7 cas sur 10 de leucémie myéloïde chronique et dans 2 cas de leucémie granulocytaire aiguë, ce qui semblait indiquer une grande fréquence de cette anomalie au cours des syndromes myélo-prolifératifs, la confirmation ne paraît pas en avoir été apportée. Quelques autres travaux font état de la présence du facteur rhumatoïde au cours de leucémies, sans que le type de celles-ci soit précisé [4, 21]. En outre, la présence de myélofibrose au cours des maladies auto-immunes, notamment du lupus érythémateux disséminé [26] a été rapportée.

Ainsi, et malgré les réserves formulées ci-dessus, l'association de désordres immunitaires et de splénomégalie myéloïde avec myélofibrose ne semble pas seulement fortuite. S'il en est bien ainsi, quelle est la signification de ces anomalies immunitaires? Les rapports de causalité semblent pouvoir s'établir de diverses manières. Ou bien les anomalies immuni-

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Capacity of Rat Haemopoietic Colony-Forming Units to Produce Differentiated Progeny

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Abstract. The capacity of rat haemopoietic colony forming units (CFU) to produce differentiated progeny has been investigated. The colony differential of primary colonies in the spleen was dependent on whether spleen or bone marrow was used as the source of the CFU. However, both bone marrow and spleen derived colonies showed increases in the proportion of undifferentiated colonies and in the number of both surface and total colonies as the time of colony development was extended. On the other hand the colony differential of primary colonies in the bone marrow was independent of the source of the CFU. Colonies in the bone marrow did not change in number or histological type as the period of colony development was extended. Since differentiation of CFU was more complete in the bone marrow compared to the spleen, it is suggested that the bone marrow in rats represents the better site to study stem cell differentiation. Calculations based on the results of individual colony transplant experiments suggest that approximately 30% of rat bone marrow CFU do not form colonies in the spleen because they differentiate.

Key Words
Bone marrow colonies
Colony forming units
Haemopoiesis
Spleen colonies
Stem cell differentiation

Although the rat has often been used to investigate the effect of cytotoxic agents on haemopoietic tissue [1], very little is known about the properties of haemopoietic stem cells in this species. Since it has been shown [2, 3] that there are differences between rats and mice in the response of haemopoietic colony forming units (CFU) to certain proliferation-dependent cytotoxic agents, it would be useful if the properties of these cells in rats were more fully known.

The proliferation rates of transplanted rat bone marrow or spleen

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Although the rat has often been used to investigate the effect of cytotoxic agents on haemopoietic tissue [1] very little is known about the properties of haemopoietic stem cells in this species. Since it has been shown [2 3] that there are differences between rats and mice in the response of haemopoietic colony forming units (CFU) to certain proliferation-dependent cytotoxic agents it would be useful if the properties of these cells in rats were more fully known

The proliferation rates of transplanted rat bone marrow or spleen

CFU have been reported [4] The population doubling times (T_d) of these cells in both the bone marrow and spleen were essentially similar to those observed for haemopoietic CFU in mice It was shown that the cell cycle time (T_c) of rat bone marrow CFU was similar (7–10 h) in both the bone marrow and spleen although CFU in the bone marrow had a T_d of approximately 36 h compared to 18 h for the same population in the spleen Using these values of T_c and T_d calculations suggested that 35–45% of rat bone marrow CFU in the spleen 4 days post transplantation were lost by differentiation per CFU division compared to 70–75% in the bone marrow at a similar level of recovery The difference in the proportion of CFU lost by differentiation in the spleen and bone marrow could explain the different CFU population doubling times in these two organs [4]

Two detailed reports [5, 6] have considered the histology of bone marrow-derived colonies in the spleens of rats treated with cytotoxic chemicals In both studies it was evident that a high proportion of rat spleen colonies consisted either entirely of cells, arbitrarily termed 'undifferentiated', which were too immature to classify as belonging to any one haemopoietic cell line, or of a mixture of these cells with recognizable erythroid cells ['erythrocyte variant', 6] Data were also presented [5] which showed that unlike spleen colonies in mice, rat spleen colonies increased in number as the time for colony development was extended from 7 or 8 to 12 days This change was associated with a shift towards a higher proportion of colonies consisting of undifferentiated cells The present report extends these observations and investigates the histology of bone marrow and spleen derived colonies in both the spleen and bone marrow of rats treated with cytotoxic agents From the transplantation of individual bone marrow-derived spleen colonies a further estimate of the proportion of CFU lost through differentiation has been obtained

Materials and Methods

Female pure line Wilkie Hooded rats aged 6–8 weeks were used throughout

Bone marrow cell suspensions were prepared by flushing out the marrow with TC199 from both femurs of up to 5 donor rats and aspirating the suspension through a 19 gauge needle to obtain a separated cell suspension [5] Spleen cell suspensions were prepared by forcing spleens through stainless steel gauzes of gradually decreasing mesh size and finally through a 19 gauge needle The number of cells in each suspension was determined using haemocytometers Dilutions

in TC199 were carried where necessary to obtain the required number of cells in a 1-ml volume

Haemopoietic colonies derived from bone marrow or spleen CFU were obtained in both the bone marrow and spleen of groups of at least 5 rats treated with 30 mg/kg busulphan and 4 mg/kg aminochlorambucil according to the previously reported method [7]. The mean number of endogenous colonies/spleen at 8.5 days after aminochlorambucil (corresponding to 8-day colonies) in all the experiments now reported was less than 0.5. This number increased to 1.2 ± 0.2 colonies/spleen 12.5 days after aminochlorambucil (corresponding to 12-day colonies). The mean number of endogenous colonies/femur was 1.0 ± 0.9 at 8.5 days after aminochlorambucil and did not increase in number up to 12.5 days after aminochlorambucil.

The number of surface spleen colonies at various times after the transplantation of bone marrow or spleen cells was assessed using a × 2 magnification hand lens after fixation of the spleen in Carnoy's fixative for 1–2 h [5]. The total number and histological type of colony in both the spleen and femoral bone marrow were obtained from microscopical analysis of 5 µm thick serial, longitudinal sections stained in haemotoxylin and eosin [5, 7]. Except for megakaryocytic colonies (where 5 megakaryocytes or more were considered a colony) more than 100 cells were classified as a colony which was considered pure, i.e., consisting of one cell line only, unless it contained more than 10% of another cell type [5, 7].

In colony transplant experiments, individual 10-day bone marrow-derived spleen colonies were dissected out of the spleens, under a dissecting microscope, imprinted onto a microscope slide for subsequent staining and histological analysis, aspirated up and down through a 19-gauge needle to obtain a single cell suspension, counted and the whole of each individual colony transplanted into a secondary recipient prepared for spleen colony development. 'Controls' received a similar number of cells to those in a colony (q.v.) prepared, as above, from parts of colony-containing spleens which contained no colonies. The numbers of both surface and total colonies were determined 8 days after the secondary transplant. In none of the 13 'controls' did the number of colonies in either the bone marrow or spleen exceed the number of endogenous colonies (see above). Although routine assays for CFU were carried out 8 days post transplantation, 10-day colonies were used as a source of CFU in these experiments since they are larger and, hence, more easily dissected from the spleen than colonies at 8 days [5].

All results are expressed in terms of the mean ± the standard error calculated from the data obtained from the recipient rats and to give an indication of the variation within the groups of recipients [2]. Results are judged significantly different from control if $p < 0.05$ calculated using Student's t test.

Since the capacity of bone marrow or spleen derived CFU to produce colonies in both the bone marrow and spleen has been investigated, the following abbreviations are used: BM/S for primary bone marrow-derived colonies in the spleen and BM/BM for primary bone marrow-derived colonies in the bone marrow; S/S for primary spleen-derived colonies in the spleen and S/BM for primary spleen-derived colonies in the bone marrow. Secondary colonies produced by the transplantation of 10-day BM/S colonies are designated SC/BM for secondary colonies in the bone marrow and SC/S for secondary colonies in the spleen.

Results

Primary colonies in the spleen Previous results [5], which have been confirmed in the present study, showed that 4×10^5 nucleated bone marrow cells produced 10–12 surface BM/S colonies 8 days post transplantation. Approximately 4×10^6 nucleated spleen cells were required to produce a similar number of surface S/S colonies (table I) As the time of colony development was extended, the number of both surface and total colonies increased but only at 12 days was there a significant difference between the number of surface and total colonies (table I)

The proportions of both 8-day BM/S and 8-day S/S colonies belonging to a particular haematological cell line (the 'colony differential') were independent of the number of bone marrow or spleen cells transplanted [see also 5]. 8-day S/S colonies contained significantly higher proportions of erythroid+undifferentiated and megakaryocytic colonies and a significantly smaller proportion of undifferentiated colonies than 8-day BM/S colonies (tables I and IV) [5].

Table I The numbers and histological lines of spleen-derived spleen (S/S) colonies at different times after the transplantation of 4×10^6 spleen cells

Day after 4×10^6 spleen cells	Number of spleens	Mean number of colonies/spleen		Histological line, % of total number of colonies					
		surface	total	E	G	Meg	U	E+U	others
8	10	11.3±0.6[1]	18.5±3.5	22±2	0	4±1	24±4	52±3	0
9	7	17.6±2.0	16.0±4.0	16±8	0	3±2	27±7	54±4	0
10	5	20.4±2.0	17.0±4.0	11±6	0	6±2	31±5	52±5	0
11	6	22.4±0.9	24.1±2.3	4±1*	0	13±1*	50±2*	33±2*	0
12	10	23.2±0.8	31.2±2.7*	3±1*	0	13±4*	43±3*	41±2*	0

E = Erythroid, G = granulocytic, Meg = megakarocytic, U = undifferentiated, E + U = erythroid + undifferentiated, others = any mixed colonies (other than E + U) consisting of two or more cell lines

* Significantly ($p < 0.05$) different from the proportions of 8-day colonies.

The difference between the numbers of surface colonies and total colonies is significant ($p < 0.05$) only at 12 days post transplantation

[1] ± standard error

Table II. The colony differentials of colonies in the femoral bone marrow 8 days after the transplantation of 0.5–2.0 × 10⁴ bone marrow cells or 5.0–20.0 × 10⁴ spleen cells

Number of cells transplanted	Total number of femurs	Total number of colonies examined	Histological line, % of total number of colonies					
			E	G	Meg	U	E+U	others
0.5–2.0 × 10⁴ bone marrow cells (BM/BM colonies)	36	204	59 ± 6	26 ± 4	9 ± 5	0	0	2 ± 2
5.0–20.0 × 10⁴ spleen cells (S/BM colonies)	36	180	63 ± 5	26 ± 3	11 ± 2	0	0	2 ± 1

For further details see figure 1 and legend to table I.

Table III. The number of 8-day colonies in secondary recipients per 10⁴ cells after the transplantation of individual 10-day BM/S colonies

Type of colony as cell source	Number of colonies transplanted	Mean number of cells per colony × 10⁶	Colonies/10⁴ cells transplanted in			Ratio of secondary colonies in spleen/secondary colonies in one femur
			spleen		one femur	
			surface	total	total	
E	8	2.9 ± 0.5	3.3 ± 1.2	3.2 ± 0.5	1.7 ± 0.3	1.9 ± 0.4
U	11	3.6 ± 0.3	0.9 ± 0.4	0.9 ± 0.1	2.4 ± 0.3	0.4 ± 0.2
E + U	11	2.3 ± 0.3	3.6 ± 0.9	3.7 ± 0.3	2.2 ± 0.3	1.7 ± 0.2
						Ratio of primary colonies in spleen/primary colonies in one femur
Normal bone marrow			25.0 ± 2.0	27.0 ± 1.5	600 ± 100†	0.04 ± 0.01
Normal spleen			2.4 ± 0.6	4.6 ± 1.0	50 ± 10†	0.05 ± 0.01

For further details, see table I.

† Obtained by extrapolation of the data in figure 1.

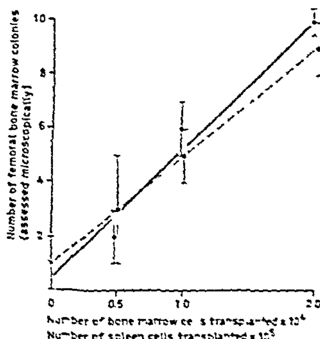


Fig. 1. The relationship between the number of colonies/femur 8 days after the transplantation of various numbers of bone marrow (circles and solid line) or spleen (squares and broken line) cells. Vertical bars indicate ± standard error.

As the time of development of S/S colonies was extended, the proportions of undifferentiated and megakaryocytic colonies increased and the proportion of erythroid and erythroid + undifferentiated colonies decreased (table I). Similar changes were seen in BM/S colonies [5] except that megakaryocytic colonies were not detected throughout the period of study.

Primary colonies in the femoral bone marrow. Approximately 10^5 bone marrow cells or 10^5 spleen cells were required to produce 5 colonies/femur 8 days post transplantation (fig. 1). Extending the time of colony development up to 12 days did not increase the number of colonies. The colony differentials of colonies in the bone marrow were independent of the numb… ells transplanted, the source, i.e., bone marrow or spleen, of the CF… and the time of colony development.

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Table II. The colony differentials of 8-day SC/S and SC/BM after the transplantation of individual 10-day BM/S colonies

Type of colony as cell source	Total number of secondary colonies examined	Histological line, % of total number of colonies					
		E	G	Meg	U	E+U	others
In the spleen (SC/S colonies)							
E	74	21±3	0	2±1*	29±4*	49±4*	0
U	36	28±8	0	3±1*	24±6*	45±11	0
E+U	94	32±5	0	1±1	20±4*	48±5*	0
Primary BM/S	>200	24±2	0	0	46±2	30±2	0
In the bone marrow (SC/BM)							
E	40	65±7	30±7	3±3	0	0	5±5
U	77	59±4	32±4	8±2	0	0	8±3
E+U	56	50±6	34±5	5±3	0	0	9±5
Primary BM/BM (from table II)	204	59±6	26±4	9±5	0	0	2±1

For legend, see table I.
* Significantly different from primary bone marrow derived colonies.

mal bone marrow (table III). The number of secondary spleen colonies produced per 10^4 colony cells transplanted more closely resembled the number produced per 10^4 spleen cells than 10^4 bone marrow cells. The number of secondary colonies/femur produced per 10^4 BM/S colony cells was less than 0.01 of the number expected after the primary transplant of 10^4 bone marrow cells. The colony differentials (table IV) of SC/S colonies were independent of the type of primary BM/S colony used as the CFU source. The differentials, more closely resembled primary S/S colonies (table I) than primary BM/S colonies (table IV) in the proportions of megakaryocytic, undifferentiated and erythroid+undifferentiated colonies. The colony differentials of SC/BM did not, however, differ significantly from primary BM/BM colonies (table IV).

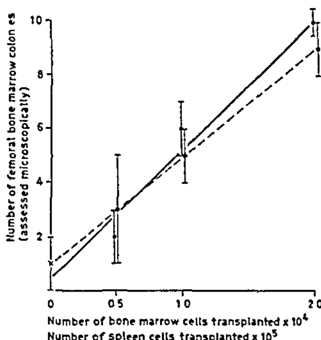


Fig 1 The relationship between the number of colonies/femur 8 days after the transplantation of various numbers of bone marrow (circles and solid line) or spleen (squares and broken line) cells Vertical bars indicate ± standard error

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Primary colonies in the femoral bone marrow Approximately 10^4 bone marrow cells or 10^5 spleen cells were required to produce 5 colonies/femur 8 days post transplantation (fig 1) Extending the time of colony development up to 12 days did not increase the number of colonies The colony differentials of colonies in the bone marrow were independent of the number of cells transplanted, the source, i e , bone marrow or spleen, of the CFU (table II) and the time of colony development

Secondary colonies in the spleen and femoral bone marrow after the transplantation of primary BM/S colonies All histological types of primary BM/S colonies were deficient in CFU/10^6 cells compared to nor-

Table IV. The colony differentials of 8-day SC/S and SC/BM after the transplantation of individual 10-day BM/S colonies

Type of colony as cell source	Total number of secondary colonies examined	Histological line, %, of total number of colonies					
		E	G	Meg	U	E+U	others
In the spleen (SC/S colonies)							
E	74	21±3	0	2±1*	29±4*	49±4*	0
U	36	25±8	0	3±1*	24±6*	45±11	0
E+U	94	32±5	0	1±1	20±4*	45±5*	0
Primary BM/S	>200	24±2	0	0	46±2	30±2	0
In the bone marrow (SC/BM)							
E	40	65±7	30±7	3±3	0	0	5±5
U	77	59±4	32±4	8±2	0	0	8±3
E+U	56	50±6	34±5	5±3	0	0	9±5
Primary BM/BM (from table II)	204	59±6	26±4	9±5	0	0	2±1

For legend, see table I.
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Table V. Values of p and ω for rat bone marrow CFU proliferating in 10-day spleen colonies

Type of colony	$\bar{K}$+SD	V	p[1]	ω[1]
Erythroid	9.5±9.6	1.01	0.75	0.33
Mixed	8.4±9.2	1.11	0.77	0.30
Undifferentiated	2.4±2.8	1.17	0.80	0.25
Mean			0.77	0.29

[1] Calculated according to the method of VOGEL *et al.* [22] using the formula applicable to the transplantation of single colonies, viz.

$$V^2 = \left(\frac{2-2p}{2p-1}\right) + \frac{1}{\bar{K}},$$

where $\bar{K}$ = average number of secondary spleen colonies, V = coefficient of variation of the colony distribution in secondary recipients $= \frac{\text{standard deviations (SD)}}{\bar{K}}$, p = probability of self renewal of CFU, ω = extinction coefficient $= \frac{1-p}{p}$

Discussion

Haemopoietic colonies in rats are similar to those in mice [8, 9] in that 10 times as many spleen cells (table I) as bone marrow cells [5] were required to produce a given number of spleen colonies. Also like mice [10] rat spleen-derived spleen colonies contain a higher proportion of megakaryocytic colonies than bone marrow-derived spleen colonies (tables I and IV). Colonies in the bone marrow of both rats (tables II and IV) and mice [11] contain a higher proportion of granulocytic colonies than colonies in the spleen.

Colonies in rats, however, differ markedly from colonies in mice in several ways. In rats (table III), transplantation of a given number of bone marrow or spleen cells produced approximately 20 times more colonies in one femur than in the spleen. In mice, twice as many colonies are seen in the spleen than in one femur [11, 12]. This species difference may be a reflection of the smaller role of the spleen in rats during haemopoietic recovery after lethal treatment and bone marrow grafting [5, 13, 14]. A large proportion of rat spleen colonies consisted of undif-

ferentiated cells while granulocytic colonies were absent (table I) [5]. It has been suggested [5] that some of these undifferentiated cells may be macrophages and the similarity between the increase in the proportion of these spleen colonies with time to the changes occurring in *in vitro* colonies has been pointed out [15]. The reasons for the absence of granulocytic colonies in rat spleens are not clear. Preparation of rats with cytotoxic chemicals rather than irradiation was not responsible since granulocytic colonies were present in the bone marrow (tables II and IV) *and the spleens of irradiated and bone marrow grafted rats*, also contained no granulocytic colonies [5]. Unlike mice [16, 17] the number of spleen colonies in rats increased with time of colony development [5] (table I) although only at 12 days was there a significant ($p < 0.05$) difference between the number of surface colonies and the total number of colonies. A large but not significant ($p = 0.1$) difference between the total number and the surface number of colonies was observed at 8 days post transplantation and such a difference might be expected if colonies, during the early stages of development, have to reach a certain critical size before they are visible on the spleen surface. The lack of a significant difference between the total number and surface number of colonies from 9 to 11 days suggest there are preferred areas for colony development just under the splenic capsule and show the increase in number is a real effect which cannot be explained only by an increase in size of 8-day colonies [5]. If this were so it would be expected that the total number of colonies would remain constant while the number of surface colonies would increase with time until it equalled the total number of colonies. CFU migration within the spleen such that one CFU can initiate more than one colony [18] is a possible explanation for the increase in both total number and surface number of colonies with time. However, if this occurs it must be larger in extent and/or proceed for much longer in rat, rather than mouse, spleens since no increase in spleen colony number in mice with time has been reported [16, 17].

The large proportion of undifferentiated colonies and the increase in colony number with time suggest that the spleen colony assay in rats is probably less useful than the equivalent technique in mice particularly to study CFU differentiation. In rat femoral bone marrow, however, the ratio of erythroid colonies to granulocytic colonies was 2:1 or 3:1 (table II) which is similar to the ratio of those colonies in mouse spleens [16]. Since less than 5% of the colonies in rat bone marrow consis undifferentiated cells (tables II and IV), differentiation of CFU pro

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Mean			0.77	0.29

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where $\bar{K}$ = average number of secondary spleen colonies, V = coefficient of variation of the colony distribution in secondary recipients

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The large proportion of undifferentiated colonies and the increase in colony number with time suggest that the spleen colony assay in rats is probably less useful than the equivalent technique in mice particularly to study CFU differentiation. In rat femoral bone marrow, however, the ratio of erythroid colonies to granulocytic colonies was 2:1 or 3:1 (table II) which is similar to the ratio of those colonies in mouse spleens [16]. Since less than 5% of the colonies in rat bone marrow consisted of undifferentiated cells (tables II and IV), differentiation of CFU progeny

was almost complete Furthermore, the observations that the colony differentials and number did not change with time of colony development supports the hypothesis [15] that the rat bone marrow could be used to study CFU differentiation Such a study is, however, handicapped by the considerable technical work involved (e g decalcification) in preparing bone sections for histological analysis of the marrow(s)

The transplantation of individual 10-day BM/S colonies produced secondary colonies in both the spleen (SC/S) and femoral bone marrow (SC/BM, table III) The mean number of SC/S colonies produced per BM/S colony was higher than the mean number observed after the transplantation of 10-day BM/S colonies in mice [19] and all of the rat colonies contained at least one CFU Primary undifferentiated rat BM/S colonies contained fewer CFU than BM/S colonies containing recognizable erythroid cells (table III) Unlike the situation in mice [19] the colony differentials of SC/S colonies were independent of the histological type of primary colony (table IV) The number of SC/BM colonies formed per 10^6 cells transplanted was considerably lower than the number of primary colonies obtained from a bone marrow or spleen cell graft (table III) although the colony differentials did not differ significantly from primary colonies (table IV) The relevance of these observations is not yet clear The findings that CFU/10^6 BM/S colony cells more closely resembled the number of CFU/10^6 spleen cells than CFU/10^6 bone marrow cells (table III) and the similarity of SC/S colony differentials to S/S, and not BM/S colonies, may support the hypothesis [4] that the splenic microenvironment [11] influences the capacity for self maintenance and/or differentiation of CFU 'passaged through the spleen (in BM/S colonies) Alternatively, the change in the ratio of SC/S SC/BM colonies compared to the ratio BM/S BM/BM obtained after a primary bone marrow graft may indicate the presence of two CFU populations *which preferentially seed either the bone marrow or spleen* [20]

The number of secondary spleen colonies produced by the transplantation of individual BM/S colonies (table III) allows the calculation of the probability of self renewal, p [21] and of the extinction coefficient, ω [22] of rat bone marrow CFU proliferating in the primary colonies Depending on the colony type, p ranges between 0 75 and 0 80 and ω between 0 25 and 0 33 (table V) These results indicate that in 10-day spleen colonies 25–33% of rat bone marrow colony forming cells (compared to 60–65% of mouse bone marrow colony forming cells) [22] do not form colonies because they differentiate The values for ω compare

with values of 0.35–0.45 calculated 4 days post transplantation using the relationship [23] between a CFU cell cycle time T_c of 8.3–10.3 h, estimated by the method of BLACKETT [24] and a CFU population doubling time T_d of 18 h [4] (see Introduction). The rather higher values for ω 4 days post transplantation compared to 10 days are not inconsistent with the hypothesis proposed from studies of CFU proliferation in mice [25] that a higher loss of CFU through differentiation occurs at earlier rather than later times after bone marrow transplantation.

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Distribution of Colony-Forming Cells in Mouse Bone Marrow

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Abstract. The distribution of colony forming cells in mouse bone marrow was studied using the modified exocolonizing test and the incorporation of ^{59}Fe into spleens of recipient mice. It has been found that the colony forming cells are distributed in the bone marrow of femora and lumbar vertebrae rather randomly and not accumulated near the endosteal surface and in the marrow of trabecular bone.

Key Words
Bone localization of stem cells
Bone marrow cells
Colony forming cells
Mouse bone marrow

In previously reported experiments we were interested in the effect of incorporated ^{226}Ra on the compartment size of colony forming cells in mice [3, 10]. It has been assumed that the distribution of colony forming cells in bone marrow is not uniform and that these cells may be accumulated rather near the endosteal surfaces. Such a distribution was held for one of the possible factors, which could influence the results obtained. Maas *et al.* [5] studying the bone marrow distribution in the swine skeleton, also admit that the haemopoietic stem cells may be found predominantly in the vicinity of endosteal surfaces. The hypothesis is supported to a certain extent by the interpretation of the experimental results of Patt and Maloney [8, 9], Maloney and Patt [6] in rabbits and of Fono *et al.* [2] in mice. They found that the process of regeneration after the localized mechanical depletion of femoral marrow initiates in the uncommitted mesenchymal cells of endosteal crevices and Haversian canals.

Considering that the localization of colony forming cells in bone marrow may be interesting not only from the aspect of histogenesis of hae-

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Results

The peripheral erythrocytes amounted to $10.2 \pm 0.2 \times 10^6/mm^3$ and the leucocytes to $10.9 \pm 0.7 \times 10^3$. The bone marrow cell counts are presented in tables I and II.

When mice with similar parameters were tested and cell suspensions prepared by grinding the femora and washing the cells into the suspending medium, as described in our previous experiments [10], no statistical differences were observed in colony counts and splenic ^{59}Fe uptake as well as in total femoral colony forming units (table I).

When the cells were gained both by flushing the medullary cavity (65% of total femoral marrow cells) and by grinding and washing the partially evacuated bones, cells from different sites of the femoral mar-

Table I. Colony formation, ^{59}Fe uptake in recipient spleens, and marrow cell as well as CFU counts (7 donor mice per experimental group)

Bone	Number of recipients	Age of donors, weeks	Preparation of marrow cell suspension	Number of spleen colonies per 1×10^5 marrow cells	18-hour ^{59}Fe uptake in recipient spleens after injection of 1×10^5 marrow cells, % of activity injected	Bone marrow cell counts $\times 10^6$	Total counts of CFU $\times 10^3$
Femur	18	9	BGW	23.2 ± 1.4	3.1 ± 0.2	26.2	6.1
Femur	17	14	BGW	24.2 ± 1.1	3.8 ± 0.3	25.8	6.2
Femur	22	9	MCF	25.[illegible] ± 0.9	3.1 ± 0.3	15.5	4.0
Femur evacuated by flushing	19		BGW	23.7 ± 1.0	3.4 ± 0.3	8.4	2.0
Femoral diaphysis	20	10	MCF	21.4 ± 1.2	2.7 ± 0.3	14.0	3.0
Evacuated femoral diaphysis	19		BGW	15.7 ± 1.4	2.2 ± 0.3	2.0	0.3
Femur	18	12	BGW	22.4 ± 1.2	2.6 ± 0.2	23.8	6.3
Lumbar vertebrae	16		BGW	23.7 ± 1.6	2.6 ± 0.3	7.6[1]	1.8[1]

Cellular suspensions were prepared by different techniques. BGW = bone grinding and washing. MCF = marrow cavity flushing. The data are means ± standard errors.

[1] Per one vertebra.

mopoietic stem cells but also in the case of impaired and/or regenerating marrow haemopoiesis, an attempt has been made to obtain more precise information on the distribution of haemopoietic stem cells within the mouse bone marrow through the exocolonizing test

Material and Methods

Random bred female ICR mice aged 10–14 weeks weighing approximately 27 g and caged under specific pathogen free conditions, were used The peripheral blood counts bone marrow cellularity and relative as well as total counts of colony forming units (CFU) were determined The ^{59}Fe incorporation into recipient spleens was tested concurrently

The mice were killed in groups of 7 by cervical transection The right femora and the lumbar vertebrae were dissected and cleaned Pooled marrow cell suspensions were prepared and tested as follows (1) the femora were ground in a mortar and marrow cells were washed into Tyrode s solution (first part of table I) (2) the femoral marrow was flushed out by introducing an appropriate injection needle into the proximal metaphyses and injecting Tyrode s solution repeatedly into the marrow cavities of the femora perforated on the distal articular surfaces Then the partially evacuated femora were ground and a suspension of marrow cells prepared (second part of table I) (3) both ends of the femora were separated in the *dividing line between the metaphyses and diaphyses. The diaphyseal marrow* was expelled by injecting Tyrode s solution Then the evacuated diaphyses were ground and the cells remaining on the endosteal surfaces were suspended in the used medium (third part of table I) (4) in one group of mice both femoral and vertebral marrow cell suspensions were gained by grinding and washing the bones 1 femur and 5 lumbar vertebrae per each of the tested mice (fourth part of table I) and (5) the femora were divided into three parts by cutting off the ends (metaphyses and epiphyses) and these parts were processed as described above (table II)

Nucleated bone marrow cells were counted as soon as the suspensions were prepared and then the appropriate dilution was made so that the required 1×10^5 cells to be grafted were contained in 0 2 ml The suspensions were prepared and preserved under aseptic conditions at the temperature of ice-cold water

No later than 120 min after killing the donors the cells were injected into the tail vein of syngeneic female recipients aged 10 weeks previously exposed to 800 R (HVL 1 0 mm Cu distance between focus and object 50 cm exposure rate 60 R/min) The background of endogenous spleen colonies averaged to 0 2 colony per spleen

On the 9th day, and 18 h after the intraperitoneal injection of 1 μCi of ^{59}Fe the recipient mice were killed by cervical transection The spleens were dissected and fixed in Bouin s solution The relative spleen ^{59}Fe activity was measured by means of a Packard Auto-Gamma Spectrometer and the colonies were counted independently by three observers 24 h later in a stereo-microscope at fourfold magnification The means and standard errors were computed and the statistical significances were evaluated by utilizing Student s t test

Results

The peripheral erythrocytes amounted to $10.2 \pm 0.2 \times 10^6/mm^3$ and the leucocytes to $10.9 \pm 0.7 \times 10^3$. The bone marrow cell counts are presented in tables I and II

When mice with similar parameters were tested and cell suspensions prepared by grinding the femora and washing the cells into the suspending medium, as described in our previous experiments [10], no statistical differences were observed in colony counts and splenic ^{59}Fe uptake as well as in total femoral colony-forming units (table I)

When the cells were gained both by flushing the medullary cavity (65% of total femoral marrow cells) and by grinding and washing the partially evacuated bones, cells from different sites of the femoral mar-

Table I. Colony formation, ^{59}Fe uptake in recipient spleens, and marrow cell as well as CFU counts (7 donor mice per experimental group)

Bone	Number of recipients	Age of donors, weeks	Preparation of marrow cell suspension	Number of spleen colonies per 1×10^5 marrow cells	18 hour ^{59}Fe uptake in recipient spleens after injection of 1×10^5 marrow cells, % of activity injected	Bone marrow cell counts $\times 10^6$	*Total counts of CFU* $\times 10^3$
Femur	18	9	BGW	23.2±1.4	3.1±0.2	26.2	6.1
Femur	17	14	BGW	24.2±1.1	3.8±0.3	25.8	6.2
Femur	22	9	MCF	25.7±0.9	3.1±0.3	15.5	4.0
Femur evacuated by flushing	19		BGW	23.7±1.0	3.4±0.3	8.4	2.0
Femoral diaphysis	20	10	MCF	21.4±1.2	2.7±0.3	14.0	3.0
Evacuated femoral diaphysis	19		BGW	15.7±1.4	2.2±0.3	2.0	0.3
Femur	18	12	BGW	22.4±1.2	2.6±0.2	23.8	6.5
Lumbar vertebrae	16		BGW	23.7±1.6	2.6±0.3	7.6[1]	1.8[1]

Cellular suspensions were prepared by different techniques. BGW = bone grinding and washing. MCF = marrow cavity flushing. The data are means ± standard errors.

[1] Per one vertebra

Table II Colony formation and ^{59}Fe uptake in recipient spleens after injection of 1×10^5 nucleated bone marrow cells from three portions of femoral bone marrow (7 donor mice per experimental *interval*)

Age of donors, weeks	Number of recipients in the individual groups	Number of spleen colonies after injection of marrow cells from the evaluated femoral parts			18-hour ^{59}Fe uptake in recipient *spleens after* injection of marrow cells from the evaluated femoral portions, % of activity injected			Total marrow cell counts $\times 10^6$	Distribution of marrow cells, %			Total CFU in femoral marrow $\times 10^3$	Distribution of CFU in femoral marrow, %		
		proximal end	diaphysis	distal end	proximal end	diaphysis	distal end		proximal end	diaphysis	distal end		proximal end	diaphysis	distal end
10	13, 13, 14	18.6±1.3	16.7±1.0	14.9±1.0	1.7±0.2	1.4±0.2	1.3±0.2	31.9	12.8	56.5	30.7	5.1	14.8	56.6	26.8
17	*14, 12, 12*	*26.4±1.9*	*22.8±1.9*	*26.3±1.5*	3.2±0.3	3.2±0.4	3.2±0.1	23.2	13.4	56.0	30.6	5.6	14.6	52.6	32.8
22	13, 12, 14	23.8±1.3	26.4±2.0	22.1±1.8	2.5±0.3	2.2±0.1	2.3±0.2	28.5	15.4	53.0	31.6	7.0	14.9	56.8	28.3
29	15, 16, 12	31.0±2.3	25.5±1.6	24.2±1.3	3.2±0.3	2.7±0.3	2.5±0.2	24.7	15.0	58.7	26.3	6.4	17.8	57.7	24.5
54	15, 13, 14	26.0±1.2	23.0±1.9	22.0±1.6	3.2±0.3	2.9±0.3	2.3±0.2	29.5	17.0	54.5	28.5	6.8	19.0	54.0	27.0

Total marrow cell and CFU counts in the femoral cavity and the distribution of these cells in the investigated femoral parts. The data are means ± standard errors

row might be present in each of these suspensions. However, statistical differences were found in neither the colony counts nor the relative ^{59}Fe activity in recipient spleens.

The femoral shafts were taken as the only source of marrow cells in a further group of mice and, as in the previous series, two separate cell suspensions were prepared by two different techniques. Most bone marrow cells (87.5%) were expelled by injecting the suspending medium and then the cells localized at the endosteal surfaces were removed by grinding and washing the empty diaphyses. In the second case, the yield of spleen colonies per 1×10^5 bone marrow cells was significantly lower ($p < 0.01$) than after the administration of cells prepared by the classical procedure, although no statistical difference was found in splenic ^{59}Fe uptake.

In the next group of animals, the number of femoral and vertebral CFU per 1×10^5 bone marrow cells was compared. As in the uptake of ^{59}Fe in recipient spleens no differences were registered (table I).

In the last experimental series, the exogenous colonies and ^{59}Fe incorporation in recipient spleens were investigated after grafting marrow cells gained separately from femoral ends and femoral shafts (table II). The mice were killed at 10, 17, 22, 29 and 54 weeks of age. The number of colonies and splenic ^{59}Fe incorporation were repeatedly found to be somewhat higher after the injection of marrow cells from the proximal ends than after grafting the cells from the shafts and/or distal ends of the femora. The data obtained could be compared with the paired *t* test and no significant statistical differences were observed either between the relative CFU counts in proximal ends and in diaphyses or between counts of these cells in distal ends and in shafts. However, the CFU ratio in the proximal end of the bone was significantly higher than in the distal end ($p < 0.05$). Comparing the ^{59}Fe incorporation into the recipient spleens, a statistical difference was observed only in one case. The ^{59}Fe uptake in spleens of recipient mice grafted with marrow cells from the proximal parts of the femora has been found to be significantly higher ($p < 0.05$) than after injection of marrow cells from the femoral diaphyses.

Table II shows that about 16% of femoral CFU are localized in the proximal parts, 55.5% in the diaphyses and the remaining 28.5% in the distal ends of the femora. Total femoral marrow cells are distributed similarly: the proximal ends contain approximately 14.7, the diaphyses 55.7 and the distal ends 29.6% of nucleated marrow elements. The total

CFU in femoral marrow, calculated from the relative CFU counts and the cellularity of investigated marrow portions, had grown till the 22nd week of age and then remained at the reached level

Discussion

The distribution of CFU was studied not only in the different parts of the femora but also in two different bones and an attempt was made to determine relative counts of these marrow elements near the endosteal surfaces. The data were obtained by using the modified colony-forming test as previously described. It was considered important from the aspect of evaluation that the comparable results were not statistically different, when cell suspensions from the same parts of the bone marrow were prepared by different techniques.

The marrow cavities of long bones may be divided into three parts differing from each other in the arrangement of the bone. Metaphyseal and epiphyseal segments of the medullary cavities are characterized by cancellous structures not present in the shafts. These structural differences can influence the specialized bone marrow stroma which is, according to TAVASSOLI and CROSBY [11], essential to haemopoietic proliferation. As suggested by LOBUE *et al.* [4] femoral epiphyses and shafts in rats appear to differ in their vascular patterns, the cellular ecology they provide, and their relative accessibility to experimental agents. Local differences in the haemopoietic microenvironment influencing the CFU counts can be anticipated.

The process of marrow regeneration was studied by BRÅNEMARK *et al.* [1], MALONEY and PATT [6] and FONG *et al.* [2] in rabbits and mice and it was concluded that cells with haemopoietic potential are present in osseous tissue and on the endosteal surfaces. The endosteal surfaces measured per volume unit of bone marrow must be significantly larger in bones characterized by trabecular structures than in diaphyseal cavities. In other words, the contact of marrow with the osseous tissue is larger in the first case. Thus, relative counts of colony forming units might be higher near the endosteum than in the rest of the bone marrow volume. Such a supposed accumulation implicates a higher CFU ratio in the cavities of trabecular bone rather than in the shaft.

It has been shown that the colonies after injection of 1×10^5 metaphyseal and epiphyseal marrow cells are not significantly higher when com

pared with those derived from diaphyseal marrow cells. Likewise, no difference has been found between the relative counts of colonies derived either from the lumbar or femoral marrow cells. Nevertheless, colony counts after the injection of marrow cells from the proximal ends of femora are repeatedly slightly higher than after grafting cells from other sites of the femoral cavity. Because of the low content of marrow in the proximal ends, the comparison of the distribution of total CFU and marrow cells in the investigated femoral portions does not reflect very expressively the above observation. It is supposed that this phenomenon does not result from the relationship between bone marrow and endosteal surfaces, but that other factors, such as circulating blood volume, must be taken into consideration.

An attempt was made to prove the accumulation of CFU in marrow adjacent to bone, yet, low colony counts, perhaps also influenced by the handling procedure, were found after injection of bone marrow cells gained by washing the ground shafts previously evacuated.

When corresponding data were compared, the uptake of ^{59}Fe in recipient spleens was in most cases in good correlation with colony counts. Thus, some evidence is also given that striking differences of erythroid differentiation due to the bone marrow localization do not exist.

This study has shown that the colony-forming cells, which have all or some properties of haemopoietic stem cells, are distributed rather randomly in the mouse bone marrow and are not accumulated in the vicinity of the endosteum and in the marrow of trabecular bone. Nevertheless, it cannot be excluded that differences due to the arrangement and localization of the bone marrow may be found under other than physiological conditions.

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Concurrent Infectious Mononucleosis and Acute Myelocytic Leukemia

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Abstract. The case of a patient with infectious mononucleosis and acute myelocytic leukemia is presented. The course of the leukemia seems not to have been influenced beneficially by the infection. The total survival time was 11 months. The data of this patient are discussed in relation to those of other patients with concurrent infectious mononucleosis and acute leukemia, reported in the literature.

Key Words
Infectious mononucleosis
Acute leukemia

The concurrence of infectious mononucleosis and acute lymphocytic leukemia might have a beneficial effect on the course of the latter disease as suggested by Stevens *et al.* [13]. They collected data from 7 cases, either published [2, 10-12] or unpublished, or cases followed by themselves. The age of the patients at the onset of leukemia varied between 6 and 18 years. The total time of survival amounted to 18 months (death attributed to bacterial infection without systemic relapse), 38, 44, and more than 33, 51, 80 and 102 months, respectively. Serum samples from long term survivors with acute lymphocytic leukemia without a history of concurrent infectious mononucleosis were found to have a geometric mean titer of anti-EBV antibodies higher than various control groups [13]. Another 4 cases, ranging in age from 3.75 to 13 years, were added by Lerdman *et al.* [6], bringing the total number of reported cases of concurrent infectious mononucleosis and acute lymphocytic leukemia up to 11. The total survival times were 22 months, more than 13 months (relapse), and more than 11 and 12 years. The diagnosis in the patient who survived more than 11 years remains presumptive, as heterophil antibody titers were not obtained. In the other long term survivor, infectious

mononucleosis preceded acute leukemia by 3 months TAYLOR [14] reported on 4 patients aged 35–50 years with acute monocytic leukemia and infectious mononucleosis In one of them, the infection had been acquired accidentally, in the others by the deliberate injection of acute-stage infectious mononucleosis serum Despite transient improvement of the clinical state, total survivals were short (2, 5, 6 and 13 months) The clinical course may have been influenced by the limited therapeutic possibilities at that time We know of only one patient in the literature [3] with concurrent acute myelocytic leukemia and infectious mononucleosis The total survival of this 21-year-old man was 11 months The authors felt that their data did not warrant speculation as to how one disease might have altered the course of the other

In this paper, another case of concurrent acute myelocytic leukemia and infectious mononucleosis is reported

Case Report

A 14 year-old girl was admitted on February 8, 1967, with a history of 2 weeks of dizziness and pallor Examination revealed a pale, febrile girl with a few small lymph nodes in the cervical region and a slightly enlarged spleen She weighed 40 kg Hemoglobin 6.2 g/100 ml hematocrit 19%, white blood cell count 7,400 mm³ with 70% blasts, 2% neutrophils and 28% lymphocytes platelet count 17,500/mm³ The peroxydase reaction was positive in 52% of peripheral white blood cells A predominance of blasts was seen in the bone marrow aspirate, some of them with Auer rods A heterophil titer was negative The diagnosis of acute myelocytic leukemia was made Partial remission resulted from treatment with prednisone, 50 mg orally daily and intravenous vincristine, once weekly in doses climbing from 0.6 to 4 mg Hematologic values are given in table I The patient was dismissed in good general condition in April, 1967 with only a few blasts in the peripheral blood Maintenance therapy consisted of 6-mercaptopurine, 50–100 mg orally daily She remained well until June 15, when fever and nonproductive coughing started Readmission followed on June 21 On physical examination the findings were as described above The temperature was 40 °C. Hematological data included a white blood cell count of 600 mm³ without blasts, a platelet count of 100 000 mm³ which later fell to 25 000 mm³ and a bone marrow smear which was considered normal The liver function was disturbed (table I) The thorax X ray was noncontributory Because of leucopenia, 6-mercaptopurine was discontinued Ampicillin and cloxacillin were administered despite negative blood and urine cultures An intermittent fever with daily peaks up to 40 °C persisted until July 6 Afterwards there was a gradual decline of temperature to normal values reached on July 12. The liver function then improved rather rapidly On July 25 a lymphocytosis of 68% was discovered with 12% atypical lymphocytes. The heterophil titer at that time was 1 64 after guinea pig

kidney absorption. The patient was dismissed in good condition on August 4. Maintenance therapy was instituted with intramuscular methotrexate, 5–15 mg once weekly, which was the maximum tolerated dose. She remained well until January, 1968, when she relapsed. She died on April 25, 1968, despite treatment with prednisone, vincristine, and daunorubicin.

Discussion

The diagnosis of infectious mononucleosis, made in our patient, seems to be justified by the finding of a heterophil antibody titer of 1:64 after absorption with guinea pig kidney and the presence of atypical lymphocytes in a rather high number. As both signs were detected as late as 2 weeks after subsidence of a 4-week fever, it is uncertain whether they can be related to the fever. However, the heterophil titer may have been elevated before. It was not determined, as the diagnosis was not considered. Lymphocytosis and atypical lymphocytes can be expected to appear some time during the first 2 weeks of infectious mononucleosis [5, 8], if at least bacterial infection has not supervened [8]. On the other hand, BAKST and LEIBOWITZ [1] reported on a case with first appearance of significant titers of heterophil antibodies and significant numbers of atypical lymphocytes in the 7th week of illness. We wonder whether a supervening infection or the 6-mercaptopurine therapy could have postponed the appearance of atypical lymphocytes in our patient as long as 6 weeks after the onset of symptoms. An alternative possibility is that two distinct disease processes were involved, the former appearing as fever of unknown origin, the latter being infectious mononucleosis. There is some indication of a two-phase leukopenia supporting this view (table I). The first period of leukopenia can have been caused, at least partially, by 6-mercaptopurine therapy. This leukopenia may have contributed to acquisition of an infection which can have aggravated leukopenia and thrombocytopenia. After a gradual rise of leukocytes, a second decline was seen in conjunction with the signs of infectious mononucleosis. STEVENS *et al.* [13] drew attention to the brief leukopenia occurring in all their 7 cases of concurrent infectious mononucleosis and acute leukemia. It is not clear from the literature whether leukopenia is a common feature in infectious mononucleosis. A number of reports quoted by FINCH [5] indicate that slight leukopenia may develop in as many as 20% of patients, while HOAGLAND [9] found transient leukopenia in fewer than 1% of cases. We feel that any infection may cause a decline of peripheral blood cell counts in patients

Table I Laboratory data of a patient with concurrent infectious mononucleosis and acute myelocytic leukemia

Date	Peripheral blood					Heterophil titre after guinea pig kidney absorption	Serum			
	WBC/mm³	poly-morpho-nuclear leukocy-tes and bands, %[1]	blasts/ blastoid cells %	lympho-cytes, typical/ atypical %	thrombo-cytes mm³		alkaline phospha-tase[1], Bessey U	SGOT[1] Wroblew-ski U	SGPT[1],	Bilirubin[1] mg/ 100 ml
February 9	7,400	2	70 0	28 0	17,500	negative	3 7			
April 3	1,900	27	3/4	64 0	145,000					
May 12	1,700	18	1/7	52,0	280 000					
June 1	4,800	49	0 0	37,0	182,000					
June 15	onset fever									
June 21	600	40	0 0	60 0	100,000		7,9			
June 23								41	123	<1
July 4	900				42,500					11 3
July 7	1,200				25,000					
July 10		53	1,0	43,0			58 2	110	170	
July 14	2,400				140 000					
July 17							42.9	40	88	2.9
July 18	3,800									
July 24	1,600						14 4	30	57	2.3
July 25	1,900	10	1/3	56/12	112,500					
July 26						64				
July 31						16	5 1	16	23	<1
August 10	5,100	41	0 0	51/2	95,000					

[1] Normal values alkaline phosphatase, 0 8–3.5 Bessey U; SGOT, 5–40 Wroblewski U; SGPT, 5–35 Wroblewski U; bilirubin, <1 2 mg/100 ml

Table II. Clinical course of a patient with acute myelocytic leukemia and infectious mononucleosis

Onset of AML symptoms	Age at onset years	Initial therapy	Maintenance therapy	Elapsed time		Total survival months
				from AML onset to IM, months	from IM to systemic relapse, months	
1967	14	V, P	6MP, MTX	4-5.5	7-5.5	11

AML = Acute myelocytic leukemia, IM = infectious mononucleosis. V = vincristine, P = prednisone. 6MP = 6-mercaptopurine, MTX = methotrexate.

with a compromised bone marrow. This holds for patients suffering from panmyelopathy, as pointed out by WUNJA *et al.* [16] and also for those patients whose marrow capacity has been limited by chemotherapy.

As can be seen from table I, there was a considerable, though transient, increase of serum alkaline phosphatase and bilirubin. Many observations indicate the predominance of abnormal liver function in infectious mononucleosis. FUTTERWEIT [7] pointed out the frequent elevation of serum alkaline phosphatase, in many cases disproportionate to serum bilirubin values. Increases of alkaline phosphatase have been reported in cytomegalovirus mononucleosis [11], which is very closely related to infectious mononucleosis, both with regard to clinical picture and causal agent. However, elevations as high as those found in our patient can be considered quite unusual in infectious mononucleosis. The use of 6-mercaptopurine might be responsible – at least partially – for the hepatic dysfunction. It is noteworthy that jaundice, related to this drug, was frequently accompanied by leukopenia [4].

The total survival in the present patient with acute myelocytic leukemia was in line with our experience, and seems not to have been influenced by the concurrent infection (table II). Long term survivals were recorded in patients with acute lymphocytic leukemia complicated by infectious mononucleosis [6, 13]. It has to be noted that the time elapsed from the onset of leukemia to the onset of infectious mononucleosis in the patients reported by STEVENS *et al.* [13] was longest (30 and 45 months, respectively) in the two patients with the longest survival time (more than 80 and more than 102 months). In the patient reported by FREEDMAN *et*

al. [6], who survived more than 11 years, the infection supervened as late as 46 months after the diagnosis of acute leukemia. These data cast some doubt on the beneficial influence of infectious mononucleosis on the course of the leukemia in these patients, as the rather long survival periods elapsed before infectious mononucleosis supervened might already point to a favorable prognosis. On the other hand, the beneficial effect of virus infections on leukemia has been documented by several investigators [13]. WHEELOCK and DINGLE [15] observed a repeated ameliorating effect of successive inoculations with six different viruses in a patient with acute myelocytic leukemia. A virus might achieve this effect either by a direct oncolytic potential, by interfering with the hypothetical leukemia virus (for instance by interferon), or by activation of the immune response. Infectious mononucleosis is known to be accompanied by production of a wide variety of antibodies. As the causal agent, Epstein-Barr virus (EBV), seems to have an almost exclusive affinity to lymphoid cells, the blast cells of acute lymphocytic leukemia might well harbor the virus and even promote its replication. KLEIN *et al.* [9] discovered virus-specific new antigens on the surface of EBV-infected cells and associated membrane-reactive antibodies in human sera. It is conceivable that such an immunological membrane reactivity could sustain a remission by eliminating EBV-infected blast cells. These considerations seem to be accessible to investigation now. Further reports on the concurrence of the two diseases may be helpful in studying a possible effect of infectious mononucleosis on the course of acute leukemia.

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H. J. Woodliff und R. P. Herrmann: Concise Haematology. Arnold, London 1973. 216 pp., £ 3.60.

Die Autoren haben sich die schwere Aufgabe gestellt, eine leicht verständliche und doch umfassende Einführung in die Hämatologie zu schreiben, welche sich von der Physiologie und Biochemie bis zur klinischen Diagnostik und Therapie erstreckt. Sie richtet sich in erster Linie an Medizinstudenten oder praktizierende Ärzte. Dagegen ist der Text – laut Vorwort – nicht für «postgraduate students» bestimmt. Das dürfte allerdings ein echt englisches Understatement sein.

Nach einem kurzen Überblick über die wichtigsten Methoden folgt die Besprechung der verschiedenen *Anämien*, der *myelo-* und *lymphoproliferativen Störungen* und schliesslich der hämorrhagischen Diathesen. Das Buch schliesst mit einem Kapitel über Blutgruppen und Transfusion. Durch Weglassen allen Ballastes, klare Gliederung und Setzung von Akzenten ist ein äusserst nützliches modernes Buch entstanden, welches sein Ziel aufs beste erreicht. Der Text ist sehr klar und leicht verständlich geschrieben und durch wenige, aber gut zusammengestellte Abbildungen und Tabellen bereichert. Ebenso sorgfältig ausgewählte Literaturhinweise regen zu weiterer Lektüre an.

Das Buch, welches auch in einer preiswerten Paperback-Ausgabe zu haben ist, *kann allen Studenten und Ärzten*, welche *Zugang zur Hämatologie suchen*, *ohne* sich spezialisieren zu wollen, aufs wärmste empfohlen werden. Für die Gestaltung des Unterrichtes dürfte es manchem Fachhämatologen gute Anregungen geben.

U. Bucher, *Bern*

Varia

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La Fondation Claude Adolphe Nativelle pour l'art et la médecine institue un prix international destiné à souligner la valeur de recherches fondamentales cliniques ou thérapeutiques sur les maladies cardiovasculaires. Les lauréats seront choisis sans distinction de nationalité, selon deux critères: la qualité et l'originalité de leurs travaux, la contribution que ceux-ci auront apportée au rayonnement de la langue et de la pensée scientifique françaises.

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Stimulation and Reactivity of Leukaemic Cells in Acute Myeloid Leukaemia

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Abstract. ^{14}C-thymidine incorporation and cytotoxicity to a target cell were used to assess response of peripheral blood leucocytes from patients during the course of acute myeloid leukaemia. Studies were made after mixed culture with allogeneic lymphocytes and stimulation by mitogens. The leukaemic cells were found to respond to both types of stimulus but, just prior to relapse, a fall in reactivity following mixed culture was observed whereas response to mitogens at this time was usually enhanced. The leukaemic leucocytes were found to provoke both marked cytotoxicity and significantly increased ^{14}C-thymidine uptake in normal lymphocytes after mixed culture. The degree of reactivity provoked altered during the course of the disease.

Key Words
Acute myeloid leukaemia
Cytotoxicity
Leukaemic cell stimulation
Mixed cell culture
PHA

It seems likely that lymphocyte destruction of chromium labelled target cells following stimulation by mitogens or histoincompatible cells may be more closely related to cell mediated immunity than incorporation of radioactive nucleotides. In previous studies, we have shown that the challenge to normal lymphocytes by leukaemic cells will activate both these mechanisms [8, 11] and since the response following mixed cell culture (MLC) appears to be greater than could be explained by differences of HL-A antigens alone it may be viewed as indirect evidence of the presence of leukaemic antigens [1, 3, 4, 10, 14]. Further evidence for the presence of such antigens is the atypical response of leukaemic cells when exposed to the alloantisera used in tissue typing studies [6]. In our own work we have observed apparent changes in the HL-A phenotype during the course of the disease in addition to the presence of extra antigens on the blast cells [7].

In the present series of experiments we have followed the reactivity of normal lymphocytes after culture with leukaemic cells at varying stages of the disease and assessed both [14]C-thymidine incorporation and cytotoxicity The behaviour of the leukaemic patient's own peripheral blood leucocytes in response to phytohaemagglutinin (PHA) and to the challenge of allogeneic lymphocytes has also been examined In an effort to determine whether there was any change in the ability of the circulating leukaemic cells to react to a given antigenic challenge, cultures were performed at intervals using cells from the same normal and leukaemic individuals

Materials and Methods

Cells from 11 patients with acute myeloid leukaemia (AML) and 3 with chronic myeloid leukaemia in blastic crisis were studied in one way mixed leucocyte cultures with lymphocytes from healthy donors The leukaemic cells were assessed both as stimulating and responding cells and in addition their response to the mitogenic action of PHA was recorded In 7 patients with AML and in 2 of those with chronic myeloid leukaemia we were able to perform several cultures during the period of the disease from the initial acute phase through remission and in relapse In 4 cases of AML, cells from the same normal and leukaemic pairs were cultured on each occasion To minimise the effects of drug or transfusion therapy on our results, cultures were prepared at a time when no drugs were being administered or at least 7 days after a course of treatment or blood transfusion However all 3 of the patients with chronic myeloid leukaemia were receiving cytotoxic drugs at the time of our cultures

Progress of the disease state was assessed by routine haematological examination of peripheral blood and bone marrow films Remission was defined as the presence of less than 5% of blast cells in the bone marrow, and an absence of these cells from the peripheral blood

Separation and culture of cells from peripheral blood The techniques of separation and preparation of cells the culture procedure the assessment of reactivity and expression of results are outlined below but have been described in detail elsewhere [8 9]

Leucocytes were obtained from defibrinated blood after sedimentation with plasmagel' without removal of neutrophils or remaining red blood cells The cells were resuspended in Eagles MEM supplemented with antibiotics and 10% fetal calf serum and the count adjusted to 2 0-2.5 $\times$ 10[6] lymphocytes or mononuclear cells per ml One way cultures were set up after irradiation of the stimulating cells with 4 000 r from a cobalt-60 orbitron Equal numbers of reacting and stimulating cells were mixed together and cultured for 3 days in an atmosphere of 5% CO_2 in air with appropriate controls

Assessment of cytotoxicity and [14]C thymidine incorporation Cytotoxicity was assessed in one set of cultures by the addition of [51]Cr labelled Chang liver cells

and determination of the percentage isotope released after a further 18 h incubation. The final results were expressed as the percentage increased cytotoxicity. Levels greater than 5% were regarded as significant [8]. Thymidine incorporation was measured by the addition of ^{14}C-thymidine to the other duplicate set of cultures and incorporation was stopped after a further 18-hour incubation. Aliquots of the cultures were then processed on filter paper discs and counts performed using a liquid scintillation counter. The counts obtained were related to the viable cells in the culture at the time ^{14}C-thymidine incorporation was assessed. Results were expressed as cpm/10^6 viable cells and any value in excess of 1 000 cpm above the control value was regarded as a significant increase [9].

Stimulation of leukaemic cells with PHA. 5 ml of sterile freeze-dried extract of Phytohaemagglutinin (Welcome Reagent grade Lot K 3660) was reconstituted in 30 ml of phosphate buffered saline (Oxoid) and distributed in 0.5 ml aliquots which were then stored at −20 °C. To induce transformation of cells in culture 10 µl of this suspension was added to every 1.0×10^6 lymphocytes or mononuclear cells. The cultures were assessed at 3 days for both cytotoxicity and ^{14}C-thymidine uptake in the same manner as that described in the mixed leucocyte reactions.

Results

Leukaemic Cells as Stimulators in Mixed Culture

Irradiated cells from patients with acute myeloid leukaemia were found to provoke increased cytotoxicity in normal lymphocytes when compared with results obtained in one-way cultures between normal individuals, $p = 0.05 <> 0.025$ (fig. 1). The leukaemic cells were also found to stimulate normal cells to an increased uptake of ^{14}C-thymidine and compared with the response between normal cells, $p = 0.025 <> 0.02$, but there was considerable variation in the degree of this response (fig. 2).

Alterations in the Reactivity of Normal Lymphocytes to Cells from the Same Leukaemic Patients during the Course of the Illness

In some cases the stimulatory effect of leukaemic cells to normal lymphocytes was studied at various times during the disease process using the same leukaemia normal pairs on each occasion. The results are given in table I and show great variability unlike repeated cultures between pairs of normal individuals which vary only by 10% in this system. The stimulatory capacity of the leukaemic cells seems to bear no relation to the progress of the disease or to the number of blast cells present in the peripheral blood of the leukaemic patient. Cytotoxicity and uptake of ^{14}C-thymidine also showed no correlation one with an-

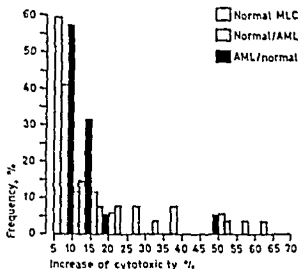


Fig 1 Assessment of cytotoxicity after mixed lymphocyte culture (236 observations)



Fig 2 Assessment of ^{14}C thymidine uptake after mixed cell culture (235 observations)

other, significant levels of reaction being observed in one assessment in the absence of such a finding in the other

Leukaemic Cells as Reactor Cells

1 Response to PHA (table II) In cultures assessed by ^{14}C-thymidine incorporation significant response by peripheral white cells from leukaemic patients was observed both in remission and relapse Reactiv-

Table 1 Reactivity of the same normal cells to leukaemic cells in mixed culture (MLC) during course of illness

Interval from onset of illness, months	MLC % increased cytotoxicity to target cell system	MLC increase in ^{14}C-uptake above 1 000 cpm	Blast cells present in leukaemic blood mm^{3}	Comments
1 At presentation	26	16,102	8 736	Initial presentation
4	0	2,758	nil	Remission
5	12	2,772	nil	Remission
5½	26	4 985	255	Early relapse
9	6	12,027	825	Relapse
10	25	113	50 052	Relapse
12	23	3 094	35 476	Relapse
2				
6	2	4 056	nil	Relapse
6½	5	1 192	nil	Relapse
7	20	0	nil	Relapse
9	0	581	1,352	Relapse
9	0	–	621,320	Relapse
3				
4	9	6,571	nil	Remission
6	12	674	nil	Early relapse
7	0	167	nil	Relapse
11	0	13 091	21	Relapse
13	0	0	nil	Relapse
4 At presentation	0	0	nil	Patient treated but no remission obtained
1	35	5 517	nil	
2	0	1,376	18,352	

ity varied from low to excessively high incorporation of labelled thymidine and contrasted with minor fluctuation found with normal lymphocytes in response to this mitogen. The greatest values were seen at the time of relapse

When *leukaemic leucocytes* were stimulated to cytotoxic activity by the action of PHA the degree of increased cytotoxicity observed was rarely higher than that produced by stimulating normal leucocytes, although, once again, the greatest response was usually found at the time

Table II The response of peripheral blood leucocytes from patients with acute myeloid leukaemia to PHA

Interval from onset of illness, months	% increased cytotoxicity to target cell system	Increase in ^{14}C-uptake above 1,000 cpm	Comments
1 At presentation	3	–	No remission
3	89	110 448	
4	243	104,611	
4½	0	175	
2			
4	149	23,150	Relapse
5	33	12,954	Relapse
3			
4	102	25,879	Remission
6	–	111,336	Early relapse
7	91	8,059	Relapse
18	70	0	Relapse
4 At presentation	5	0	
1	171	41,255	Relapse
2	3	6,883	Relapse
5			
3	146	3,719	Remission
5	213	11,472	Remission
6	129	111,653	Relapse
6½	115	64 496	Relapse
7	0	0	Relapse
7¼	139	15,567	Relapse
9	–	0	Relapse
6 At presentation	15	2,016	Relapse
3	258	51,539	
7			
4	165	19,698	Remission
6	129	23,939	Remission
7	157	51,358	Remission
9	155	986	Remission
9½	250	11,480	Early relapse
13	106	352,978	Early relapse
14	103	28,768	Early relapse
16	179	40,071	Early relapse
Blastic crisis of CML			
Onset of blastic change	0	0	
3 months later	0	5,462	
Onset of blastic change	22	1,580	
2 months later	79	1,383	

of relapse. This enhanced cytotoxic effect was in general seen at an earlier stage than the increase in ^{14}C thymidine incorporation. In both instances the mitogen was effective in provoking a response even when considerable numbers of blast cells were present.

2. *Response in mixed culture.* As shown in figure 2 leukaemic cells increased their uptake of ^{14}C-thymidine after being in mixed culture with normal lymphocytes. In some cases this reactivity was considerably greater than that found in mixed cultures between normal lymphocytes, $p = 0.025 < > 0.02$, and in general was of a higher order than in cultures where leukaemic cells were used as stimulators. The cytotoxic effect of leukaemic cells shows no comparable excessive response after mixed culture with normal lymphocytes, $p = 0.50$ (fig. 1).

3. *Alterations in reactivity of leukaemic cells in PHA and lymphocytes of the same normal donor during the course of the illness.* In 4 of the patients studied, the leukaemic cells were placed in culture with lymphocytes from the same normal donor on several occasions during the course of the disease. Table III shows the reactivity of these leukaemic cells both to PHA and to the irradiated normal lymphocytes. The cytotoxic ability of the patient's leucocytes in mixed culture was reduced or lost completely just prior to and following relapse, while the effect on target cells following PHA stimulation usually persisted. In mixed cultures assessed by ^{14}C thymidine incorporation the response usually fell as the relapse progressed. As already indicated, response to PHA was variable, the highest values being observed just prior to relapse.

Unlike the observations of CHEEMA and HERSH studying chemotherapy in tumour patients [2] there was no correlation between variations in the response to cellular challenge or mitogenic stimulation and the patient's response to therapy. It will be seen from table II, however, that the cytotoxic ability of the leukaemic leucocytes is usually absent at those times when there are large numbers of blast cells in the peripheral blood, and where the ratio of other leucocytes to lymphocytes is high. In these instances there are cells present which are still able to exhibit an increased uptake of ^{14}C thymidine and respond to PHA.

Discussion

Our results indicate that leucocytes in the peripheral blood of patients with acute leukaemia are able to stimulate normal cells in one way

Table III Reactivity of leukaemic cells during the course of illness (following MLC with cells from the same normal individual, and following stimulation by PHA

Interval from onset of illness, months	MLC % increased cyto-*toxicity* to target system	MLC increase in ^{14}C uptake above *1 000 cpm*	PHA % increased cyto-*toxicity* to target system	PHA increase in ^{14}C uptake above *1,000 cpm*	Total ratio of WBC to lymphocytes	Blast cells present in peripheral blood mm^3	Comment
1							
4	16	0	165	19,698	3 1	nil	Remission
6	0	1,624	155	986	6 1	nil	Remission
6½	9	0	250	11,450	3 1	255	Early relapse
10	0	0	106	352,977	4 1	825	Early relapse
11	0	21,981	103	29,768	50 1	50 052	Early relapse
13	0	9,162	179	40 071	50 1	35,476	Early relapse
2							
6	0	8 634	129	111,653	2 1	nil	Relapse
6½	5	1,599	115	64 496	6 1	nil	Relapse
7	9	5,911	0	0	4 1	nil	Relapse
7½	6	745	139	15,561	4 1	1,352	Relapse
9	0	–	0	–	50 1	621,320	Relapse
3							
4	0	0	102	25,879	2 1	nil	Remission
6	16	191	35	111,336	0 4 1	nil	Early relapse
7	3	0	91	8 051	2 1	nil	Relapse
18	0	0	70	0	3 1	836	Relapse
4 On presentation	1	0	5	0	2 1	nil	Acute
1	14	7 403	171	41,255	2 1	nil	Acute
	0	2,502	3	6 883	14 1	18 382	Acute

mixed culture The stimulation is apparent when assessed both by ^{14}C-thymidine incorporation and cytotoxicity to a target cell, and the reactivity is often of a greater magnitude than that produced in mixed culture between normal lymphocytes Stimulation by additional 'leukaemic antigens' might be the mechanism of the enhanced response [3, 8, 14] whilst other surface changes such as the presence of 'extra' HL-A antigens [1, 4], or the uncovering of hidden antibody binding sites are alternative explanations The degree of response elicited by the leukaemic

cells appears greater when assessed by increased cytotoxicity than by ^{14}C-thymidine incorporation. This enhanced ability to provoke the development of 'killer' lymphocytes appears to be very variable when the same normal and leukaemic cells are studied at different intervals during the disease as indeed is the increased DNA synthesis.

The leukaemic cells themselves are able to respond to the challenge provided by normal lymphocytes. It appears that peripheral blood leucocytes in myeloblastic leukaemia can be stimulated to exceptional levels of DNA synthesis, whilst their cytotoxic abilities are more in line with those of normal leucocyte populations. The leukaemic cells responded to the mitogenic effect of PHA even when the majority of the cells appeared to be morphologically primitive. Again in these cultures, a greater response was observed in ^{14}C-thymidine uptake than in target cell death. It has been suggested from work involving stimulation of leukaemic cells with UV light that many primitive cells are in fact 'resting' (G_0) and potentially capable of incorporating radioactive nucleotides [13]. This might explain the high incorporation of labelled thymidine that we have observed.

Following the course of the illness it is apparent that at the time of relapse an initial high response to PHA is frequently followed by a reduced reactivity. In mixed culture a similar reduction is found and in those cultures assessed by target cell death, cytotoxic ability is sometimes lost altogether. The change may reflect a lowering of the immunological capacity but it is difficult to determine the proportion of cells which might be inactive leukaemic cells. The influence of therapy has also to be considered although we did attempt to minimise any suppressive effect by taking samples when the patient had received no therapy for at least 7 days. Since the time intervals between therapy and culture were kept reasonably constant it is unlikely that the rebound phenomenon as described by HALTERMAN and LEVENTHAL [5] has significantly affected our observations.

When the same patient is studied during the course of the illness, the variable response observed after mixed culture suggests either that the peripheral leucocyte population changes or that the reactivity of the leucocytes present diminished as the condition relapses. In contrast are the findings with PHA where reactivity persists and ^{14}C-thymidine incorporation may increase during early relapse. This might be due to an increased susceptibility to the effect of the mitogen or to the stimulation of primitive leukaemic cells which had not previously embarked on DNA

synthesis. It has been suggested that thymidine incorporation following mixed cell culture may be governed by a separate genetic locus from that determining the aggressive activity of lymphocytes following exposure to antigen and that both loci are closely linked to those determining HL-A [15]. The disparity observed in our results after mixed culture where thymidine incorporation was compared with cytotoxicity could be regarded as further evidence for this suggestion. In the experiments described here it is not possible to determine which peripheral leucocytes are the reacting cells, and it is likely that to achieve cytotoxicity a certain minimum number of lymphocytes is required Despite these problems, it is clear that there are circulating cells in the blood of patients with acute myeloblastic leukaemia which are able to mount a reaction in mixed culture as measured by ^{14}C-thymidine uptake and which can also exert a cytotoxic effect on a target cell system. The demonstration of this potential favours the idea that specific tolerance has developed to the leukaemic cells rather than inhibition of the body's defensive mechanism [12]. Could the increased reactivity we have observed just prior to a relapse be due to a response to changing surface antigens or a 'new' leukaemic cell line which only later is accepted by the immune system? It might be that in advanced disease cytotoxic drug therapy aggravates the illness by tending to inhibit the immunological defence mechanism to a greater extent than it halts the proliferation of the malignant cells.

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Poly(A)-Containing RNA Molecules in Electrophoretically Separated Fractions of Rapidly Labeled Nuclear RNA from Unstimulated and PHA-Stimulated Human Lymphocytes

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Abstract. Agarose polyacrylamide gel electrophoresis allows detailed resolution of the high molecular weight RNA labeled in small human lymphocytes after several hours of incubation *in vitro* with a radioactive precursor. Several discrete peaks, corresponding to S values of 32, 35-36, 40-42, 45, 50, 54-55, 58-60, 70-75, and 90 were consistently observed. In PHA stimulated lymphocytes, no discrete fraction slower than the 45S RNA was identified when the cells were incubated for periods longer than 15 min. However, the relative occurrence of molecules containing poly(A) in fractions slower than 45S RNA was around 20% in both unstimulated and stimulated cells. Gel electrophoresis of poly(A)-rich RNA from unstimulated lymphocytes showed a heterogeneous distribution.

Key Words
Acrylamide electrophoresis
Lymphocytes
Nuclear RNA
PHA
Poly(A) sequences

Radioactive RNA extracted from human small lymphocytes incubated for several hours with labeled RNA precursors is mainly of nonribosomal type sedimenting faster than 28S RNA [11]. This RNA is characterized by a very low degree of methylation and by a high efficiency of RNA-DNA hybrid formation, so that it was concluded that human small lymphocytes synthesize mainly heterodisperse DNA-like RNA [11]. Competition hybridization experiments were unable to detect synthesis of RNA sequences after PHA stimulation different from those synthesized in unstimulated cells [11] although PHA stimulation seems characterized by a marked increase in both ribosomal RNA [11] and informational poly(A)-rich RNA [9]. In HeLa cells, poly(A) sequences appear in heterogeneous nuclear RNA before they appear in cytoplasmic messenger RNA, a finding consistent with the hypothesis of a precursor-product relationship between the former and the latter [1]. To better characterize the heterogeneous nuclear

dl ng characteristics of such gels are greatly improved by the addition of agarose as shown by Peacock and Dingman [6] A minor modification of the method described by these investigators was used throughout this work

RNA (50–100 μg) dissolved in buffer (NaCl 0 05 M NaEDTA 0 001 M pH 6 2) was placed on gels 0 6 cm in diameter and 7–12 cm long 1 36% acrylamide and 0 1% bisacrylamide with 0.5–1% agarose were the final concentrations In the ma jority of our experiments electrophoresis was prolonged to the point that bromophenol blue added to make the sample clearly visible and migrating somewhat more slowly than the 4S RNA had run out of the gel The time of run was usually 2–4 h at 3 mA/tube When the run was over the gels were extruded and scanned at 260 mμ in a Joice & Loebl apparatus To evaluate total RNA radioactivity the gels were sliced in 1 mm slices, then the slices were incubated for 1 h at room tempera ture with 1 ml of NCS solubilizer and diluted to 10 ml with toluene phosphor Ra dioactivity was measured in a Packard Tricarb scintillation spectrometer

To recover RNA for poly(A) assay groups of several slices corresponding to the various size ranges were left for 12 h at 4 °C in 3 ml of different buffer depend ng on the method of assay to be used

Assay of RNA molecules containing poly(A) The assay of poly(A) rich RNA molecules was based on the ability of these molecules to bind to nitrocellulose fil ters in buffer with high salt concentration [4] and to form stable duplexes with po ly(U) immobilized on glass fiber filters [10]

To evaluate the binding of labeled RNA to nitrocellulose filters 2 ml of buffer (0 01 M tris pH 7 4 0.5 M KCl 1 M $MgCl_2$) containing the RNA recovered from the gel were diluted to 10 ml with the same buffer and then filtered at room tempera ture through Millipore filters (HA 0 45 μm M llipore Filter Corp., Bedford Mass) previously soaked in the same salt solution The filters were then washed twice with 10 ml of this salt solution dried and counted in toluene scintillation mixture Input values were obtained by drying 0.5 ml aliquots of the RNA solution on Millipore filters previously soaked in high salt buffer and then counting

To evaluate the binding of labeled RNA to glass fiber filters containing poly(U) RNA in gel slices was solubilized in 3 ml of binding buffer (0 01 M tris KCl pH 7.5, 0 12 M NaCl) 2 ml of this solution were brought to 10 ml with the same buffer, then filtered at 2 ml/min through glass fiber filters (Whatman, GF/C 2.5 cm) on which poly(U) had been previously immobilized by the technique of Sheldon *et al* [10] The filters were washed with 20 ml of binding buffer at 25 °C, followed by 20 ml of ice cold 5% TCA and 10 ml of 95% ethanol, then dried and counted

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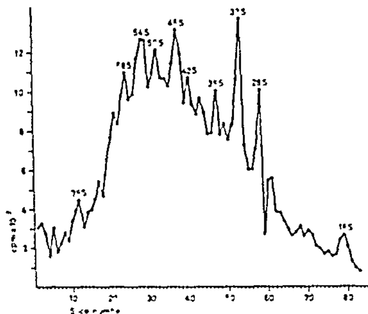
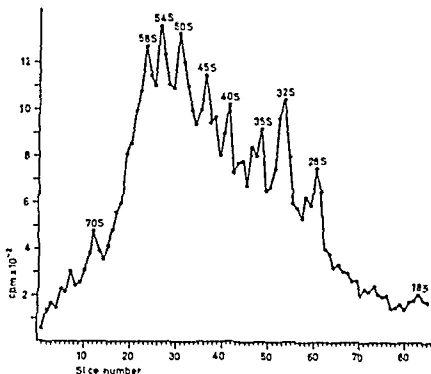


Fig 1 Polyacrylamide gel electrophoresis of whole cell RNA extracted from human small lymphocytes incubated with ³H 5-uridine (10 μCi/ml) for 6 h. Electrophoresis lasted 180 min at 3 mA/gel. After the electrophoretic run, the gel was scanned at 260 mμ and sliced in 1-mm slices. Nominal S values were assigned on the basis of the linear relationship between the logarithm of the molecular weight of a given RNA component and its relative electrophoretic mobility. The S values were therefore calculated from the known S values of the two RNA components of the ribosomal subunits (18S and 28S)

fact that electrophoresis of labeled RNA extracted from isolated nuclei gives a quite similar pattern (Fig 2). Several peaks of radioactivity were observed in all our experiments. A first group was formed by discrete fractions corresponding to S values of 32, 35–36, 40–42, 45. A second group of fractions was formed by molecules migrating more slowly than the 45S RNA. Five discrete peaks corresponding to S values of 50, 54–55, 58, 60, 70–75, and 90 were observed in the majority of our experiments. In spite of the variability in degree of separation and the relative amounts of these peaks, their width was fairly constant and similar to that of known monodisperse RNA species such as 45S and 32S preribosomal



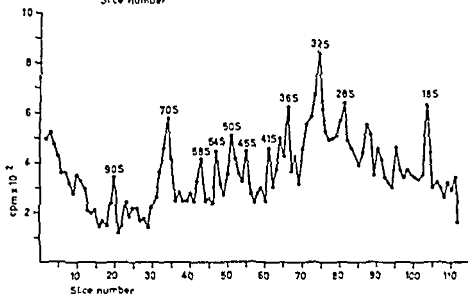


Fig 2 Electrophoretic profile of newly synthesized nuclear lymphocyte RNA. The cells were incubated with ^{3}H uridine (10 μCi/ml) for 6 h Nuclei were separated as described in 'Methods', and extracted together with enough unlabeled KB cells to give the required 28S and 18S RNA as markers. Electrophoretic conditions as described in figure 1

Fig 3 Electrophoresis of labeled whole cell RNA extracted from PHA stimulated lymphocytes incubated for 12 min with ^{3}H uridine (20 μCi/ml) Run for 200 min at 3 mA/gel

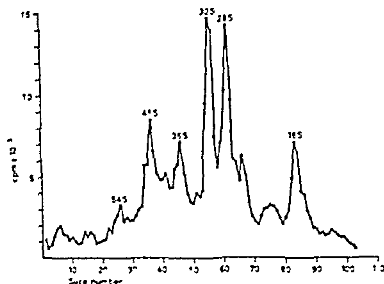


Fig. 4. Labeling pattern obtained by electrophoresis (200 min, 3 mA/gel) of whole cell RNA extracted from PHA-stimulated lymphocytes incubated with ^3H-uridine (10 μCi/ml) for 45 min.

Table 1. Binding of labeled RNA (^3H-uridine) of unstimulated and PHA-stimulated human lymphocytes to Millipore filters

RNA fraction, size range S values	Percentage of input radioactivity bound to filters					
	Small unstimulated lymphocytes			PHA-stimulated lymphocytes		
31-37	7	6	6	11	10	9
38-42	14	5	8	15	12	14
43-48	11	9	12	18	15	15
49-53	13	15	16	23	21	19
54-59	16	14	14	21	19	22
60-65	22	21	23	23	20	25
66-73	23	18	18	24	25	22
74-82	17	22	24	21	22	21
83-88	19	18	22	23	21	19
89-94	25	24	21	21	19	22
95-120	23	20	24	21	22	22

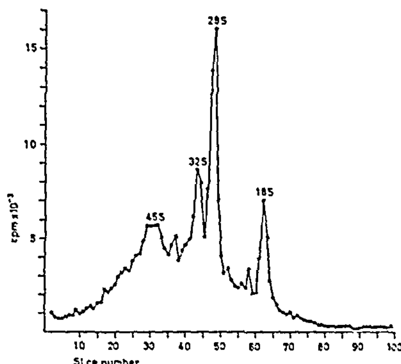


Fig. 5. Labeling pattern obtained by electrophoresis (150 min, 3 mA/gel) of RNA extracted from PHA stimulated lymphocytes incubated with ³H 5 uridine for 3 h

Table II. Binding of labeled RNA (³H-adenosine) of unstimulated and PHA-stimulated human lymphocytes to poly (U)-impregnated glass fiber filters

RNA fraction, size range, S values	Percentage of input radioactivity bound to filters					
	small unstimulated lymphocytes			PHA-stimulated lymphocytes		
31–37	4	5	6	6	7	5
38–42	4	4	6	8	8	4
43–48	6	6	7	9	6	8
49–53	6	9	8	12	10	9
54–59	10	12	9	14	13	12
60–65	12	12	9	14	12	10
66–73	12	10	12	13	9	11
74–82	11	10	14	11	10	9
83–89	10	13	13	14	12	10
90–98		11	11	12	14	8
99–120		14	12	12	9	9

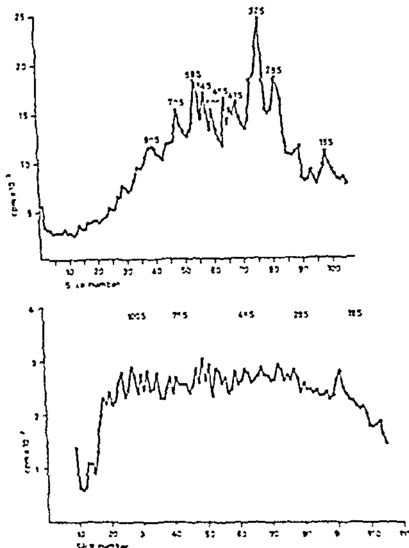


Fig. 6. a Electrophoretic profile of radioactive RNA obtained from small nuclei of lymphocytes incubated for 8 h with ³H adenosine (25 μCi/ml). *b* Electrophoretic profile of the fraction containing poly(A) of the RNA extracted from the same cells. The RNA was filtered through a Millipore filter, and 2 small discs 8 mm in diameter were cut from the filter and put on top of a gel column. The discs were then covered with electrophoresis buffer containing unlabeled KB cell RNA as a carrier. Run was prolonged for 4 h at 5 mA/gel.

RNA. These peaks were always inscribed on a large background of heterodisperse radioactivity with nominal S values reaching values higher than 100S.

In PHA-stimulated lymphocytes, discrete radioactive peaks corresponding to the nominal S values mentioned above were observed only when the RNA was extracted from cells incubated for periods shorter than 15 min (fig. 3). After longer incubations, no discrete radioactive fraction slower than 45S RNA was identified in electropherograms obtained either with whole cell RNA (fig. 4, 5) or with nuclear RNA.

Occurrence of poly(A). Tables I and II show the results of our assay of molecules containing poly(A) in the different electrophoretic fractions of rapidly labeled nuclear RNA extracted from the lymphocytes of three normal donors. They may be summarized as follows: (1) In the size range from 49S to 120S, 16–24% of the ^{3}H-adenylate-labeled RNA from both unstimulated and PHA-stimulated lymphocytes bind to nitrocellulose filters, whereas 8–14% bind to poly(U)-impregnated glass fiber filters; when the RNA was extracted from unstimulated lymphocytes, the most slowly migrating fractions (120–70S) constantly showed an occurrence of poly(A)-rich molecules slightly higher than that of other fractions, whereas very uniform values were obtained with RNA from PHA-stimulated lymphocytes; (2) The amount of filter-bound radioactivity was constantly lower in the fractions corresponding to S values lower than 48S.

The distribution of poly(A)-rich molecules was further studied by analyzing by gel electrophoresis the RNA recovered from the filters. Figure 6 shows the pattern obtained by electrophoresis of whole cell RNA extracted from unstimulated lymphocytes incubated for 8 h with ^{3}H-adenosine. It is apparent that, whereas total RNA radioactivity showed several discrete peaks behind the 28S RNA, the poly(A)-rich molecules recovered from the filter were heterogeneously dispersed through the gel.

Discussion

Our results confirm that the rapidly labeled RNA in normal human lymphocytes is mainly confined to the nucleus and is represented by RNA molecules migrating slower than the 28S ribosomal RNA. At variance with what is observed by centrifugation in density gradients, these molecules can be resolved in several monodisperse species, inscribed on a background of heterodisperse molecules reaching sizes up to 150S. At least 9

peaks, corresponding to S values of 32, 36, 41, 45, 50, 54, 58, 70, and 90 were repeatedly observed in our electropherograms. With the only exception of the 32S and 45S RNA, the discrete peaks were not seen studying RNA extracted from PHA-stimulated lymphocytes incubated with the labeled precursor for periods longer than 15 min. Furthermore, electrophoretic analysis of poly(A) rich RNA recovered from the filters shows the heterogeneous distribution of these molecules in both unstimulated and PHA-stimulated lymphocytes. These observations, as well as repetition of the pattern in several experiments, cast doubt on the theory that the discrete peaks are technical artifacts. Their appearance seems rather due to the low turnover rate characterizing the slowly migrating RNA of unstimulated lymphocytes.

Some of the peaks observed, corresponding to 32S, 35S, 41S, may be identified as intermediate products of processing of the 45S ribosomal precursor RNA. Their existence has been repeatedly demonstrated in different types of cells [8, 12]. The finding of a lower occurrence of molecules containing poly(A) in this region of the electropherogram is in keeping with this interpretation, since the ribosomal precursor RNA lacks poly(A) segments [9]. As far as the fractions slower than the 45S RNA are concerned, an analysis by agarose acrylamide gel electrophoresis has been performed by NIEMAN and HENRY [5]. These investigators have observed discrete fractions corresponding to 90, 75, 68, and 50S. By study of base composition and hybridization efficiency, they were also able to suggest the presence of different populations of RNA molecules in various regions of the gel columns. However, in our experiments the study of the distribution of the molecules containing poly(A) did not show any heterogeneity in the different segment of the gel. In the fractions behind 45S RNA, the relative occurrence of molecules containing poly(A) was quite similar to that found by GREENBERG and PERRY [3] in HnRNA of L cells. No significant difference was noted in the relative occurrence of molecules containing poly(A) before and after PHA stimulation. Although no definite conclusion can be reached, this observation supports the idea that PHA stimulation does not involve any change in the ratio between 'structural' and 'nonstructural' RNA sequences synthesized in normal lymphocytes.

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Site of Action of Desferrioxamine in Removing Iron in Normal and Pathological Conditions

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Abstract. The iron content in the organs of normal, anaemic and iron-overloaded rats was determined. The urinary iron excretion was not different in anaemic rats compared with normal rats. It was significantly increased in overloaded rats in comparison with normal rats. The iron decrease in liver, spleen, brain, kidney and muscle in highly anaemic rats was significant, while the iron increase in liver, heart, and muscle in iron-overloaded rats was evident in comparison with normal rats. After treatment with desferrioxamine the iron content of the organs was decreased in normal rats but not in anaemic or overloaded rats. The removal of iron in overloaded rats seems to be difficult.

Key Words
Anaemic rats
Desferrioxamine
Iron deficiency
Iron storage

According to some investigators [1–4] there is a significant increase in urinary iron excretion following parenteral administration of desferrioxamine (DFO) in cases of iron-storage diseases in comparison with normal iron stores. This indicates that DFO-induced urinary iron excretion correlates with the size of iron storages. Other investigators are in doubt about this correlation [5]. Despite extensive use in cases of storage diseases, the DFO site of action in removing iron remains obscure. The purpose of our investigations was to check the chelating capacity of DFO in normal and pathological conditions in order to get some information about the origin of urinary iron excretion induced by DFO. In part I of these papers [6] we described our investigations concerning the distribution and excretion pattern of ferrioxamine in rats. The injected ^{59}Fe ferrioxamine was partly excreted in the urine with a lower specific activity.

In this report, we will describe the investigations on the site of DFO

[1] The technical assistance of Mr. P. Backhuys is gratefully acknowledged.

Table 1 Iron content in the organs (expressed as μmol iron/g dry tissue) and in

	Normal rats (n = 15)		p value[2]	Anaemic rats (n = 5)	
	mean	extreme values		mean	extreme values
Liver	7.6	9.3–5.9	<0.0005	2.6	3.0–2.2
Spleen	34.8	45.7–23.9	<0.0005	7.3	8.4–6.2
Heart	5.3	6.5–4.1	0.25<p<0.30	5.1	5.8–4.4
Kidney	7.6	8.8–6.4	0.25<p<0.30	5.5	6.2–4.8
Brain	2.8	3.6–2.0	<0.0005	1.7	1.9–1.5
Muscle	2.5	3.3–1.7	0.10<p<0.15	3.0	4.1–1.9
Urine, 24 h	15 10^{-3}	28 10^{-3}–2 10^{-3}	0.25<p<0.30	13 10^{-3}	19 10^{-3}–7 10^{-3}

[1] Significance is calculated according to Student's t-test
[2] Normal rats compared to anaemic ones

action and the availability of iron in the different organs for DFO in normal and pathological conditions

Materials and Methods

Adult Wistar male rats, weighing 250–300 g, were used. Desferrioxamine methanesulphonate (Desferal®, Ciba-Geigy) was supplied in sterile vials containing 500 mg crystallized powder; prior to use, this was dissolved in isotonic saline. Urinary measurements were performed by the method of TAVENIER *et al.* [7]. The basic principle is that the Fe(III) is quantitatively extractable with methylisobutylketone (MIBK) in strong acid solution. Adding water to the MIBK layer, the Fe(III) is transferred into the water phase. After removal of the MIBK layer the desired chemicals are added to form the Fe-CNS complex, this can be measured spectrophotometrically.

The total non-haem iron content in the organs was determined following the *wet destruction method*. The iron content was measured spectrophotometrically at 535 nm [6]. In order to compare the action of DFO in normal rats, in anaemic rats, and in those with iron-storage disease, we created some pathological conditions. One group of rats was made anaemic by repeated phlebotomy to haematocrit values of 0.20 l/l by withdrawing a total of 10 ml within 4 days.

Another group of rats was made highly anaemic by withdrawing 30 ml of blood in portions of 10 ml within a 4-day interval. In a third group of rats we introduced large quantities of iron to exceed their normal total body content. Therefore, we gave parenteral injections of iron dextran, equivalent to 40 mg iron (4×10 mg).

The rats were sacrificed after administration of DFO and the organs were analyzed for iron content. The rats were kept in metabolic cages, and the urine was carefully collected in iron-free glassware.

urine (expressed as µmol iron/24 h) in normal, anaemic and iron-overloaded rats[1]

p value[3]	Highly anaemic rats (n = 5)		p value[4]	Overloaded rats (n = 10)	
	mean	extreme values		mean	extreme values
< 0.0005	1.0	1.1–0.9	< 0.0005	22.1	25.6–17.6
< 0.0005	7.1	8.1–6.1	0.10 < p < 0.15	37.8	45.9–29.7
0.15 < p < 0.20	5.0	5.4–4.6	< 0.0005	8.0	9.6–6.4
< 0.0005	1.2	1.5–0.9	0.05 < p < 0.10	7.1	8.5–5.7
< 0.0005	1.0	1.2–0.8	< 0.5	2.8	3.5–2.1
< 0.0005	0.1	0.13–0.07	< 0.0005	10.7	13.0–8.4
0.25 < p < 0.30	1.3·10⁻⁴	1.9·10⁻⁴–7·10⁻⁵	< 0.0005	3.0·10⁻³	4.4·10⁻³–1.6·10⁻³

[3] Normal rats compared to highly anaemic ones.
[4] Normal rats compared to overloaded ones.

Parenteral injections of Jectofer® (iron-sorbitol complex) were given with identical iron amount (4×10 mg) and with larger iron amounts (3×40 mg) than the iron-dextran injections. The effect of different doses of DFO on urinary iron excretion and iron content in organs was checked. Standard error of the method is less than [illegible]%.

Results

In order to obtain an insight into the behavior of DFO in normal and pathological conditions, it is necessary to know the iron content in different organs of the normal rat. These values were compared with those obtained from rats with iron storage diseases, from anaemic rats, and from extremely anaemic rats. As table I shows, there is a significant decrease in iron content of the liver, spleen, and brain in anaemic and highly anaemic rats, while the decrease for the latter group is also significant in kidney and muscle. The iron level in iron loaded rats is significantly increased in liver, heart, and muscle.

The effect of a single dose of 36 µmol DFO on the iron content of normal rats is shown in table II. The same dose of 36 µmol DFO was given to anaemic and iron loaded rats. The results are given in tables III and IV.

In order to learn whether a large amount of DFO could mobilize more iron from rats with iron-storage diseases, we injected 360 µmol DFO into rats overloaded with iron dextran. The results are shown in table IV.

Table II Iron content (expressed as μmol iron/g dry tissue) in the organs of normal rats treated with a single dose of 36 μmol DFO[1]

	Normal rats (n = 15)		Normal rats treated with 36 μmol DFO (n = 15)		p value
	mean	extreme values	mean	extreme values	
Liver	7 6	9 3– 5 9	5 3	6 1– 4 5	0 0125<p<0 025
Spleen	34 8	45 7–23 9	30 0	35 5–24 5	0 005<p<0 01
Heart	5 3	6 5– 4 1	4 6	5 8– 3 4	0 005<p<0 01
Kidney	7 6	8 8– 6 4	5 0	5 8– 4.2	<0 0005
Brain	2.8	3 6– 2.0	1 4	1 8– 1 0	<0 0005
Muscle	2.5	3 3– 1 7	1 4	1 8– 1 0	<0 0005
Urine, 24 h	$15\ 10^{-3}$	$28\ 10^{-3}$–$2\ 10^{-3}$	$100\ 10^{-3}$	$138\ 10^{-3}$–$62\ 10^{-3}$	<0 0005

[1] Iron content in urine is expressed as μmol iron/24 h, p < 0 05 is significant

Table III Iron content (expressed as μmol iron/g dry tissue) in the organs of anaemic rats treated with 36 μmol DFO[1]

	Anaemic rats (n = 5)		Anaemic + 36 μmol DFO (n = 5)		p value
	mean	extreme values	mean	extreme values	
Liver	2 6	3 0–2.2	2.6	3 0–2.2	p<0 5
Spleen	7 3	8 4–6 2	7 1	8 2–6 0	0 35<p<0 40
Heart	5 1	5 8–4 4	4 6	5 1–4 1	0 05<p<0 10
Kidney	5 5	6 2–4 8	3 2	3 7–2.7	p<0 0005
Brain	1 7	1 9–1 5	1 7	1 9–1 5	p<0 5
Muscle	3 0	4 1–1 9	1 9	2.2–1 6	0 0125<p<0 025
Urine, 24 h	$13\ 10^{-3}$	$19\ 10^{-3}$–$7\ 10^{-3}$	$15\ 10^{-3}$	$28\ 10^{-3}$–$2\ 10^{-3}$	p<0 05

[1] Iron content in urine is expressed as μmol iron/24 h

On the other hand, we injected rats with Jectofer These overloaded rats were treated with various doses of DFO The results are shown in tables V and VI

Discussion

The differences in iron content of normal rats and of rats with iron-storage diseases are obvious (table I) The iron decrease in liver, spleen,

Table II. Iron content (expressed as µmol iron/g dry tissue) in the organs of overloaded rats (40 mg iron as iron-dextran complex) without DFO, with a single 36 µmol DFO injection, and with a single 360 µmol DFO injection

	Overloaded rats with iron dextran (n = 10)		p level[1]	Overloaded rats with iron dextran + 36 µmol DFO (n = 10)		p level[2]	Overloaded rats with iron dextran + 360 µmol DFO (n = 10)	
	mean	extreme values		mean	extreme values		mean	extreme values
Liver	22.1	26.6–17.6	0.25 p 0.30	21.4	25.7–17.1	0.25 < p 0.30	18.2	22.8–13.6
Spleen	37.8	45.9–29.7	0.10 p 0.15	41.0	49.4–32.6	0.10 p 0.15	35.3	42.9–27.7
Heart	8.0	9.6–6.4	0.15 p 0.20	7.6	9.0–6.2	p<0.5	8.0	9.4–6.6
Kidney	7.1	8.5–5.[illegible]	p<0.5	7.1	8.4–5.8	p 0.5	7.1	8.5–5.7
Brain	2.8	3.5–2.1	p 0.0005	2.0	2.4–1.6	p<0.0005	2.1	2.5–1.7
Muscle	10.7	13.0–8.4	0.0005 < p 0.0025	8.8	10.4–7.2	p<0.0005	6.4	7.8–5.0
Urine 24 h	30·10[illegible]	44·10[illegible]–16·10[illegible]	p 0.005	125·10[illegible]	159.5·10[illegible]–90.5·10[illegible]	p 0.0005	2.630·10[illegible]	3.283·10[illegible]–1.976·10[illegible]

1 Overloaded rats compared to rats overloaded and treated with 36 µmol DFO
2 Overloaded rats compared to rats overloaded and treated with 360 µmol DFO

Table V Iron content (40 mg iron in the form of iron sorbitol complex) in the organs of iron/24 h) without DFO, with a single 36 µmol DFO injection a

	Overloaded rats with iron sorbitol (40 mg iron) (n = 5)		p level[1]	Overloaded rats + 36 µmol DFO (n = 5)	
	mean	extreme values		mean	extreme values
Liver	17.6	19.5–15.7	0.30<p<0.35	17.1	18.9–15.3
Spleen	26.3	28.6–24.0	0.40<p<0.45	26.0	28.2–23.8
Heart	7.2	7.9–6.5	0.25<p<0.30	7.0	7.4–6.6
Kidney	14.7	16.9–12.5	0.35<p<0.40	14.4	16.1–12.7
Brain	2.6	2.9–2.3	0.05<p<0.10	2.4	2.6–2.2
Muscle	3.5	4.1–2.9	0.25<p<0.30	3.3	4.0–2.6
Urine, 24 h	4.5	5.3–3.7	0.40<p<0.45	4.4	5.2–3.6

[1] Overloaded rats compared to rats overloaded and treated with 36 µmol DFO
[2] Overloaded rats compared to rats overloaded and treated with 360 µmol DFO

Table VI Iron content (3 × 40 mg iron in the form of iron-sorbitol complex) in the organs iron/24 h) without DFO, with a single 36 µmol DFO injection a

	Overloaded rats with iron sorbitol/3 × 40 mg iron (n = 5)		p level[1]	Overloaded rats + 36 µmol DFO (n = 5)	
	mean	extreme values		mean	extreme values
Liver	17.0	18.1–15.9	0.35<p<0.40	16.8	17.8–15.8
Spleen	26.0	27.9–24.1	0.25<p<0.30	25.4	27.1–23.7
Heart	10.0	10.2–9.8	<0.5	10.0	10.3–9.7
Kidney	16.3	16.8–15.8	0.30<p<0.35	16.2	16.5–15.9
Brain	2.3	2.4–2.2	<0.5	2.3	2.4–2.2
Muscle	3.3	3.6–3.0	<0.5	3.3	3.6–3.0
Urine, 24 h	4.5	5.2–3.8	<0.0005	2.0	2.2–1.8

[1] Overloaded rats compared to rats overloaded and treated with 36 µmol DFO
[2] Overloaded rats compared to rats overloaded and treated with 360 µmol DFO

brain, kidney, and muscle is significant for the anaemic and highly anaemic rats. The iron increase in liver, heart, and muscle is evident for overloaded rats in comparison with normal rats. Table II shows, that the iron content of the various organs in normal rats treated with DFO is de-

overloaded rats (expressed as µmol iron/g dry tissue) and in urine (expressed as µmol single 360 µmol DFO injection, and repeated 360 µmol DFO injections

p level[a]	Overloaded rats + 360 µmol DFO (n = 5)		p level[a]	Overloaded rats + 10 × 36 µmol DFO (n = 5)	
	mean	extreme values		mean	extreme values
0.15 < p < 0.20	16.6	18.3–14.9	0.20 < p < 0.25	16.8	18.4–15.2
0.005 < p < 0.01	22.3	24.4–20.2	0.025 < p < 0.05	23.4	25.7–21.1
0.25 < p < 0.30	7.0	7.5– 6.5	< 0.5	7.2	7.6– 6.8
0.35 < p < 0.40	15.0	17.0–13.0	0.45 < p < 0.475	14.8	16.9–12.7
0.10 < p < 0.15	2.4	2.6– 2.2	0.10 < p < 0.15	2.4	2.6– 2.2
0.05 < p < 0.10	3.0	3.6– 2.4	0.35 < p < 0.40	3.4	4.1– 2.7
< 0.0005	14.0	16.4–11.6	0.40 < p < 0.45	4.4	5.3– 3.5

[a] Overloaded rats compared to rats overloaded and treated with 10 × 36 µmol DFO

of overloaded rats (expressed as µmol iron/g dry tissue) and in urine (expressed as µmol single 360 µmol DFO injection and repeated 10 × 36 µmol DFO injections

p level[a]	Overloaded rats + 360 µmol DFO (n = 5)		p level[a]	Overloaded rats + 10 × 36 µmol DFO (n = 5)	
	mean	extreme values		mean	extreme values
p > 0.5	17.0	18.1–15.9	0.45 < p < 0.475	16.9	18.6–15.2
0.15 < p < 0.20	25.1	26.8–23.4	0.40 < p < 0.45	25.8	27.6–24.0
0.20 < p < 0.25	10.1	10.3– 9.9	0.10 < p < 0.15	10.2	10.5– 9.9
0.30 < p < 0.35	16.2	16.5–15.9	p > 0.5	16.3	16.7–15.9
p > 0.5	2.3	2.4– 2.2	0.05 < p < 0.10	2.2	2.3– 2.1
0.15 < p < 0.20	3.1	3.6– 2.6	p < 0.5	3.3	3.7– 2.9
p < 0.0005	9.6	10.2– 9.0	p < 0.0005	1.9	2.2– 1.6

[a] Overloaded rats compared to rats overloaded and treated with 10 × 36 µmol DFO

creased. The urinary iron excretion is significantly increased for normal rats in the first 24 h following a parenteral injection of DFO. As was expected, the iron decrease cannot be shown in anaemic rats treated with DFO (table III). In rats overloaded with iron dextran, only the iron con-

tent of brain and muscle can be decreased by injections of DFO (table IV) The urinary iron excretion is significantly increased when DFO is given Iron from parenterally administered iron dextran (stored in reticulo-endothelial cells) may not be easily available for DFO Not much iron in the iron dextran complex seems to be available for DFO [8] Therefore, it is useful to overload rats with another Jectofer iron compound in order to investigate whether this iron is easily available to DFO As tables V and VI show, there is no DFO effect on the iron content of these overloaded rats This is a rather remarkable result

DFO has a great influence on the iron content of normal rats However, this influence cannot be shown in cases of iron storage diseases Probably the iron is stored in cells and components which give no iron to DFO Therefore, use of DFO in iron-storage disease therapy is open to discussion

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Nature of the Antigammaglobulin Activity in Cryoglobulinemic Disorders

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Abstract. In mixed cryoglobulinemias, peculiar antigenic determinants of the IgG component were shown. Furthermore, in some mixed IgA-IgM-IgG cryoglobulinemias, we have found in the IgA component an anti-IgM activity. These results suggest the presence of antibodies against new antigenic determinants which appear on immunoglobulin molecules following their reaction with the specific antigen. Further evidence for this hypothesis is supplied by the study of cryoprecipitates isolated from hyperimmune animal sera: in these, we have demonstrated, in addition to the antigen used for the immunization, antibody activity against both this antigen and the animal's IgG.

Key Words
Antigammaglobulin activity
Antigenic determinants
Cryoglobulinemia
Immunoglobulins
Serum proteins
Waldenström's disease

There are two basic alternatives to explain the origin of the antiglobulin activity in cryoglobulinemic disorders: (1) random mutation of antibody producing cells; (2) a normal immunological response caused by IgG modifications, following their combination with the specific antigen. This latter possibility is by now thought to be the most probable [19, 25]. The formation of an antigen-antibody IgG complex would lead to an unfolding of the IgG molecule and to the exposure of antigenic determinants previously hidden [7-9, 10]. Against these there would be a normal antibody response of IgM or, more rarely, IgA or IgG type, resulting in an IgM-IgG or IgA-IgG or in a simple IgG cryoglobulinemia [6].

In an attempt to obtain experimental evidence supporting this proposition, we followed different pathways: (a) demonstration, through the production of a specific antiserum, of antigenic determinants in cryoglobulinemic IgG, absent in the pool of normal IgG; (b) demonstration of an-

ti-IgM activity in sera of mixed IgA-IgM-IgG cryoglobulinemia. If the above proposition is correct, these sera will present also an anti-IgM activity, supported by the persistence of circulating IgM-IgG complexes, where IgM are antibodies bound to the specific antigen; (c) investigation on cryoprecipitates in hyperimmune animal sera. According to our working hypothesis, these should contain the antigen used for the immunization, antibodies against this antigen, and antiglobulin activity.

Materials and Methods

Cryocrit. The amount of cryoglobulin in sera was evaluated by the cryocrit, a term indicating the volume occupied by the cryoprecipitate, expressed as volume percentage of whole serum after centrifugation for 10 min of the serum stored at 4 °C for 48 h, at 700 g and at 4 °C.

Isolation of cryoglobulins. The method described by MELTZER and FRANKLIN [11] was followed.

Analytical electrophoresis was carried out on cellulose acetate paper tris barbital sodium barbital buffer, pH 8.8, at 250 V for 20 min.

Immunoelectroosmophoresis was performed according to the method of PRINCE and BURKE [16].

Double diffusion was performed according to the method of OUCHTERLONY [13].

Immunoelectrophoresis was carried out following the micromethod of SCHEIDEGGER [18].

Immunorheophoresis. The method of VAN OSS and BRONSON [23] was applied. Commercially available antisera were used (Behringwerke and Hyland). A specific antiserum prepared by us (see below) was used to show antigenic determinants peculiar of cryoglobulinemic IgG.

Hemagglutination procedures were according to the method of BOYDEN [4], modified by STAVITSKY [21].

Estimation of sialic acid. WARREN's [27] method was followed on purified normal and cryoglobulinemic IgG.

Desialination of normal purified IgG was performed according to the method of BLUMBERG and WARREN [3], using Vibrio comma neuraminidase (Behringwerke).

Pronase digestion of cryoglobulins, isolated from sera of hyperimmune animals, was performed according to the method of BEDARIDA et al. [1].

Mild reduction of cryoglobulins isolated from hyperimmune animals. A 0.1 M 2-mercaptoethanol solution in 0.15 M phosphate buffer, pH 7.2, was added to the same volume of cryoglobulin solution. The reaction was allowed to proceed for 2 h at 37 °C, after which the mixture was dialyzed against 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl.

Two components (IgM-IgG) of cryoglobulins were isolated by gel filtration on Sephadex G 200, using 0.2 M acetic acid acetate buffer, pH 4.2.

Purification of normal IgG was obtained by chromatography on DEAE-cellulose according to the method of SOBER et al. [20].

Preparation of antiserum against cryoglobulinemic IgG. Rabbits were immunized following the method of FAHEY and MCLAUGHLIN [5] with purified IgG cryoglobulin isolated from case S.N. of table I. On electrophoretic and immunoelectrophoretic analysis, the IgG showed distinct features of polyclonality; the amount of sialic acid was 40 µg/100 mg of protein (normal 210 µg/100 mg).

Absorption of the antiserum. Antiserum was made specific by repeated absorption with a pool of normal human sera. In another experiment, the absorption was performed with hypogammaglobulinemic serum, with a subsequent loss in activity against all serum fractions except both normal and cryoglobulinemic IgG. The following preparations were also used: purified human albumin (Behringwerke), purified transferrin (AB Kabi), calf thymus DNA (Calbiochem).

Results

Demonstration of antigenic determinants in cryoglobulinemic IgG. Seven cases of mixed cryoglobulinemias were examined using our antiserum. Their serological and clinical features are reported in table I. On gel-diffusion precipitin analysis, this antiserum, absorbed with a pool of

Table I. Type and concentration of cryoglobulins

Patient	Age, sex	Diagnosis	Cryoglobulins (cryocrit, %)	IgM light chain type
O.P.	56, m	mixed idiopathic cryoglobulinemia	IgG IgM IgA (3–5)	k
O.S.	47, f	mixed idiopathic cryoglobulinemia	IgG IgM (2.5–3.5)	k
M.A.	42, m	mixed idiopathic cryoglobulinemia	IgG IgM (3–4)	k
M.J.	49, m	mixed idiopathic cryoglobulinemia	IgG IgM (3–6)	λ
M.M.	42, f	mixed idiopathic cryoglobulinemia	IgG IgM (1.7–5)	k
P.M.	[illegible], f	Waldenström's disease with mixed cryoglobulinemia	IgG IgM IgA (4–20)	λ
S.N.	62, f	Waldenström's disease with mixed cryoglobulinemia	IgG IgM (25)	λ

ti IgM activity in sera of mixed IgA-IgM IgG cryoglobulinemia. If the above proposition is correct, these sera will present also an anti IgM activity, supported by the persistence of circulating IgM IgG complexes, where IgM are antibodies bound to the specific antigen, (c) investigation on cryoprecipitates in hyperimmune animal sera. According to our working hypothesis these should contain the antigen used for the immunization, antibodies against this antigen, and antiglobulin activity.

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Absorption of the antiserum. Antiserum was made specific by repeated absorption with a pool of normal human sera. In another experiment, the absorption was performed with hypogammaglobulinemic serum, with a subsequent loss in activity against all serum fractions, except both normal and cryoglobulinemic IgG. The following preparations were also used: purified human albumin (Behringwerke), purified transferrin (AB Kabi), calf thymus DNA (Calbiochem).

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Patient	Age, sex	Diagnosis	Cryoglobulins (cryocrit, %)	IgM light chain type
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O. S.	49, f	mixed idiopathic cryoglobulinemia	IgG-IgM (2.5-3.5)	k
M. A.	42, m	mixed idiopathic cryoglobulinemia	IgG-IgM (3-4)	k
M. F.	43, m	mixed idiopathic cryoglobulinemia	IgG-IgM (3-4)	k
M. M.	42, f	mixed idiopathic cryoglobulinemia	IgG-IgM (1-[illegible])	k
P. M.	41, f	Waldenström's disease with mixed cryoglobulinemia	IgG-IgM-IgA (6-26)	k
[illegible]	62, f	Waldenström's disease with mixed cryoglobulinemia	IgG-IgM (20)	k

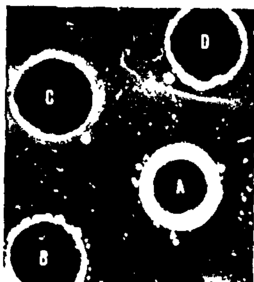


Fig. 1. Gel diffusion precipitin analysis of completely absorbed antiserum (A) normal IgG (200 mg%, B), normal serum diluted 1/10 (C) purified cryoglobulinemic IgG from patient P. M. (200 mg%, D)

Table II. Reactivity of completely absorbed anti-cryoglobulinemic IgG serum against red cells coated with normal IgG and IgG cryoglobulin

Erythrocytes coated with	Titer of hemagglutination
Normal IgG	0
IgG cryoglobulin	
G. P.	40
P. M.	20
S. N.	80

normal human sera, reacted with IgG cryoglobulin of all 7 cases, it always failed to react with normal human IgG, tested at various dilutions. An example of a precipitin reaction in agar gel is shown in figure 1. Some cases (table II) tested by hemagglutination procedures confirmed these results. When the same antiserum was absorbed with hypogammaglobulinemic instead of normal serum, it showed a reaction of partial identity between normal IgG and purified cryoglobulinemic IgG.

The completely absorbed antiserum failed also to react with the following antigens: subclasses of IgG (γ_1, γ_2, γ_3, γ_4) human IgG from

Table III. Type and concentration of cryoglobulins examined to test anti-IgM activity

Patient	Age, sex	Diagnosis	Cryoglobulin (cryocrit, %)	IgM light chain type
B.M.	78 f	Waldenström's disease with mixed cryoglobulinemia	IgG-IgM-IgA (75–82)	k
G.P.	56 m	mixed idiopathic cryoglobulinemia	IgG-IgM-IgA (3.5)	λ
I.I.	61 f	Waldenström's disease with mixed cryoglobulinemia	IgG-IgM-IgA	k
M.E.	50 f	mixed idiopathic cryoglobulinemia	IgG-IgM-IgA (5–9)	k
P.M.	57 f	Waldenström's disease with mixed cryoglobulinemia	IgG-IgM-IgA (6–26)	λ

Table IV. Inhibition of reaction of G.P. and M.E. cryoglobulins with human IgM-coated red cells by human IgM and anti-IgA serum

Patient	Titer of hemagglutination		
		sera	cryoglobulins
G.P.	in PBS	16	128
	+ IgM (20 mg%)		16
	+ anti-IgA serum		0
M.E.	in PBS	32	64
	+ IgM (20 mg%)		8
	+ anti-IgA serum		0

which sialic acid had been removed by neuraminidase and calf thymus DNA. Using specific antisera, none of the seven cryoglobulins contained a non-immunoglobulin fraction which is sometimes involved in the phenomenon of cryoprecipitation (α- and β-lipoproteins, α_2-macroglobulins, complement components).

Demonstration of anti-IgM activity in cryoglobulinemic sera. We examined 5 cases of mixed IgA-IgM-IgG cryoglobulinemia (table III). It

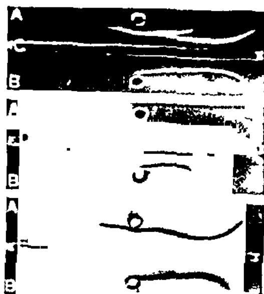


Fig. 2. Immunoelectrophoresis of patient G. P. serum and cryoglobulin. A = patient's serum, B = patient's cryoglobulin, C = anti IgA serum, D = anti IgM serum, F = anti IgG serum.

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On immunoelectrophoretic analysis, all 5 sera showed an abnormal behaviour of the *precipitin line of IgA, which appeared constantly as a* double arch. One component migrated with normal IgA morphological features, the other was more or less markedly removed towards the cathode zone, superimposable on the precipitin line of IgG in 2 cases (G. P. and M. E., fig. 2), and on that of IgM in the remaining 3 cases (fig. 3), as if it was bound to IgG or, respectively, to IgM component. When we examined the washed cryoglobulins, only this second arch was present, and it showed the same pattern already described for the whole serum (fig. 2 and 3).

Investigation on cryoprecipitates of hyperimmune animal sera. We examined the sera of 15 horses immunized against tetanus toxin. Cryoglob-

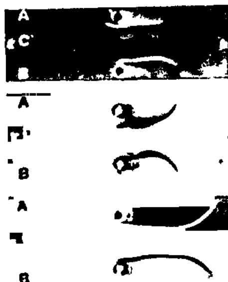


Fig. 3. Immunoelectrophoresis of patient B.M. serum. A = patient's serum, B = patient's cryoglobulin, C = anti IgA serum, D = anti IgM serum, E = anti IgG serum.

ulins were present in 13, only 2 animals with a larger quantity of cryoprecipitate were studied. Electrophoresis both of sera and of cryoglobulins did not reveal any M components. Immunoelectrophoretic analysis was performed with antitotal horse serum and anti horse immunoglobulin serum: it showed normal behaviour of the precipitin lines in the whole sera.

The cryoglobulins appeared, using this method, to consist of 2 components that showed migration and morphology of the precipitin line similar to those of IgM and IgG. Agglutination reaction of tanned red cells coated with horse IgG was performed on sera and cryoglobulins isolated from them. It revealed the presence of anti IgG activity in the cryoglobulins only (titer 1:16). On immunoelectrophoretic analysis, the same cryoglobulins reacted with purified tetanus toxin (fig. 4); mild reduction of cryoglobulin with 2 mercaptoethanol did not prevent this reaction.

In order to detect tetanus toxin eventually bound to specific antibodies in washed cryoglobulins, we employed enzymatic digestion by pronase. After this digestion, the washed cryoglobulins reacted with anti tetanus

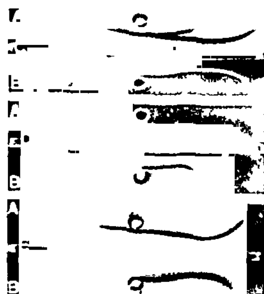


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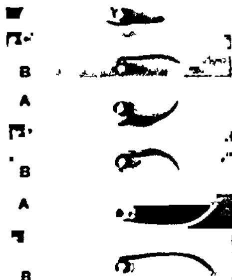


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Origin, Morphologic and Functional Characteristics of a New Lymphoid Cell Type in Irradiated Mouse Bone Marrow

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Abstract After sublethal irradiation, an immature lymphoid cell type with characteristic ultrastructure was found in the bone marrow and thymus of adult mice of different strains. Normally this cell type has only been detected in the embryo or new born. Grafting normal, adult bone marrow suppresses, while thymectomy enhances the proliferation of these cells in the irradiated organism. Immunologic and functional tests suggest that the cells have either an embryonic origin, or are derivatives of haemopoietic stem cells and function either as precursors for the immunocompetent B and T cells, or in an entirely different lymphoid system

Key Words
Bone marrow
Irradiation
Lymphoid cells

In the course of the post-irradiation recovery of the mouse bone marrow, a transitory accumulation of small mononucleated cells was observed between the 10th and 14th day after irradiation [1-3] Attempts were made to correlate this accumulation with the variation in the number of the bone marrow lymphoid cell population For this purpose, the total number of nucleated cells in the femoral bone marrow and the volume distribution of these cells were determined by a Coulter electronic particle counter, and the relative proportion of the different cell types was calculated in myelograms [4] The data indicated that the accumulation of the mononucleated cells is partly due to an increase in the number of small marrow lymphocytes In addition, a large amount of lymphocyte-like cells are present which are slightly larger and have a more basophilic cytoplasm than normal lymphocytes In subsequent experimental investigations, efforts were made to characterize more closely this cell type This report summarizes the results obtained so far

Morphology and Conditions of Development

The accumulation of mononucleated cells reaches a maximum 12 days after whole body irradiation of mice with sublethal doses of X-rays. It has been found to occur in both sexes of inbred (R3, C57Bl, CBA) as well as non inbred (Swiss albino, NMRI) strains [3–9] and also in mice maintained under germ free conditions [8]. The extent and duration of the accumulation are independent of the exposure dose in the range 80–500 r [2, 9, 10]. The phenomenon can be effectively prevented by partial protection of the bone marrow during irradiation. Similar inhibition can be obtained by intravenous injection of normal bone marrow cells up to 6 days following sublethal irradiation of the animals [11, 12].

Ultrastructural studies of the bone marrow at the time of the maximum accumulation of the mononucleated cells revealed a cell type with a particular morphology, not detectable in the cell population of normal adult marrow [13]. Its cytoplasm is rather abundant and rich in ribosomes which are either isolated or grouped in small rosettes. In the rounded nucleus the chromatine is typically aggregated in masses which are separated by numerous clear areas. The nucleolus is large and irregular in shape and the granular and fibrillar components are clearly separated. The nucleolus is also characteristically coated by a thick zone of associated chromatine. In our previous papers these cells were denoted λ cells and, for convenience, they will be referred to similarly in the present article. The features of the λ cell characterize an immature, metabolically highly active cell differing distinctly from the immature lymphoblast which has a finely dispersed chromatine, small nucleolus and thin zone of associated chromatine.

In the bone marrow foci of λ cells can be detected from the 8th post irradiation day [9]. At the time of the lymphoid like cell peak they can amount up to 50% of the total nucleated marrow cell population [13]. However the number of λ cells decreases rapidly and none are detectable 16 days after irradiation [9].

λ cells can also be found in the cortex of the thymus beginning 10 days after irradiation [14]. They reach a maximum frequency of 15% in the cortical cell population around the 14th day. Their number then decreases slowly but in contrast to the bone marrow they remain detectable until 30 days after irradiation [15].

The accumulation of λ cells in the bone marrow and thymus is inhibited by the grafting of adult bone marrow in the irradiated animals [14

15] On the other hand thymectomy 30 days before irradiation does not prevent λ cell accumulation On the contrary in thymectomized mice λ cells persist in the bone marrow for a longer period of time (up to 16 days) than in control sham-operated animals [6] *Splenectomy 5 days before ir radiation* is without any effect on λ cell accumulation [16]

While in the adult the appearance of λ cells is as a rule related to sublethal radiation exposure of the organism λ cells are detected in the marrow and the thymus of *unirradiated embryos and of new born mice* [17] The λ cells occur then in a frequency of about 5% in the nucleated bone marrow cell population Since immediately after birth the propor tion of lymphoid cells in the bone marrow is small λ cells can be the most frequent lymphoid cell type in the newborn

Immunologic and Functional Tests

The lymphoid character of the λ cells suggested by their particular morphology is further supported by the result of immunologic and func tional tests Thus treatment *in vitro* with H 2 antibodies selectively de stroys the lymphocytes and lymphocyte like elements in the irradiated re generating bone marrow as well as the lymphocytes in the normal mar row [18 19] This indicates that in regard to the H 2 antigen coating λ cells resemble normal lymphocytes Cortisone in doses which kill a large proportion of the lymphocytes in the normal marrow also effectively in hibits λ cell development when injected into irradiated animals [20 21]

Autoradiographs of bone marrow pulse labelled with tritiated thymi dine 11 days after sublethal irradiation showed that in the lymphoid group of cells about 40% of the large cell types and less than 1% of the small cell types incorporate the isotope within 1 h [9] Up to 40 h after la belling only an insignificant variation occurred in the percentage of the la belled large cells but a gradual increase was noted up to about 30% in the proportion of labelled small cells Since as discussed above λ cells constitute the majority of the large lymphocyte like cells around the 12th post irradiation day this indicates on the one hand that λ cells are ac tively proliferating and that on the other hand they can give rise to small marrow lymphocytes

The question whether λ cells represent a *transitional form between* the stem cells and other non lymphoid cell types cannot be answered by this experiment since proliferating cells of the erythropoietic and granu

locytic series will also incorporate the label. Conclusive data in this regard were obtained by analysis of myelograms [4]. The dividing compartment of granulopoiesis (myeloblasts, promyelocytes, myelocytes) was found to be reconstituted within 8 days after irradiation, i.e. before X cells accumulate in the marrow. Furthermore, no increase of either the dividing or maturation (metamyelocytes, polymorphonuclear granulocytes) compartments of the granulocytic series occurs in relation to the accumulation and or dissappearance of X cells. It can be concluded that X cells cannot act as granulocytic precursors.

As judged by the number of erythroblasts in the marrow, normal erythropoiesis is also resumed within 8–10 days after irradiation, i.e. before X cells accumulation [4]. This finding – together with the observation that changes in the rate of erythropoiesis induced by repeated bleeding or hypertransfusion of erythrocytes does not influence X cell accumulation – suggests that X cells are not related to the erythropoietic system either [9, 22]. This conclusion is further supported by the result of an experiment in which X cell-containing suspensions of bone marrow were labelled with radioactive iron and, subsequently, exposed to treatment with H 2 antibodies [18]. This treatment which, as discussed above, kills the X cells, did not induce any release of the isotope, thus indicating the failure of X cells to incorporate iron.

After intravenous injection into lethally irradiated mice, the spleen colony forming ability of regenerating bone marrow which contained X cells in a high proportion was found to be similar or slightly less than the ability of normal bone marrow [19, 23, 24]. When mice were irradiated sublethally and 12 days after exposed to a renewed radiation with a lethal dose with one leg shielded, the extent of the endogeneous spleen colony formation was again comparable to that found in control animals exposed solely to the lethal dose [25]. These experiments demonstrate that X cells cannot act as pluripotent haemopoietic stem cells.

The immature character and the lymphoid quality of the X cells raises the question concerning the role of these cells in the regeneration of the lymphoid system and the reconstitution of the immunologic reactivity of the irradiated organism. Thymus regeneration was studied in experiments in which X cell-containing marrow suspensions were injected intravenously into mice one day after exposure to an X ray dose which totally depletes the thymus [4]. As indicated by cell counts made at regular intervals, thymus repopulation was delayed considerably in comparison to controls injected with cells derived from normal bone marrow. This sug-

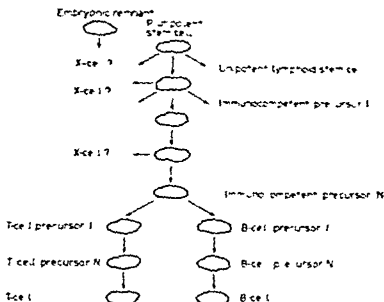


Fig 1 Schematic diagram showing the conceivable relationships of X cells to lymphopoiesis

gests that X cells do not function as precursors for thymocytes The possible relationship of X cells with thymus-derived, immunologically competent T cells was studied in experiments in which X cell-containing marrow was exposed *in vitro* to antibodies directed against the Θ antigen peculiar for T cells [Hirschi, K., unpublished data] The antibody-containing serum was prepared according to Raff [26, 27] The proportion of cells with Θ antigens, as determined in cytotoxicity tests and by immunofluorescence, was as low in the X cell-containing marrow as in the normal marrow While this indicates that X cells lack this particular antigen, obviously the possibility that X cells represent precursors for T cells cannot be excluded by this experiment

This possibility is, however, contradicted by the result of experiments in which the blastic transformation by phytohaemagglutinin was studied in the cell population of irradiated thymus at various times during recovery The reaction of the cell population derived from the thymus of whole-body irradiated animals, i e in which large amount of X cells proliferated, was during a prolonged period significantly less than the reaction of the thymic cells from animals exposed to the same dose of radiation, but in which X cell proliferation was inhibited by the shielding of

one leg [HIRSCH, K., unpublished data]. Since phytohaemagglutinin induces the blastic transformation of T cells in particular [28, 29], this result indicates that X cells cannot be identified with T cell precursors.

In order to study the possible relationship between X cells and bone marrow derived immunologically competent B cells, regenerating bone marrow suspensions and rich in X cells were tested by the Jerne plaque technique in regard to the capacity to produce haemolysins directed against sheep erythrocytes [30]. In comparison to normal bone marrow, the proportion of haemolysin producing cells was decreased in the preparations. With regard to the cooperation between B and T cells [31, 32], in complementary experiments thymus cells were added to the system as a source of T cells [BRAUNSTEINER, unpublished data]. Although the proportion of haemolytically active cells increased in the X cell-containing marrow, it was nevertheless lower than in the normal control marrow supplemented with T cells. It can be concluded that X cells are not precursors for B cells in the marrow.

This conclusion is also supported by experiments in which X cell containing bone marrow suspensions were exposed to treatment with antiserum directed against B cell antigens [HIRSCH, K., unpublished data] and prepared according to RAFF [22]. Cytotoxicity tests indicated that a smaller proportion of cells was destroyed in comparison to normal bone marrow, suggesting that X cells lack or have less B antigens than normal marrow lymphocytes.

The occurrence of X cells in the embryo and the new born and the immature character of these cells prompted investigations of the possible presence of embryonic antigens. Embryonic antiserum was prepared by immunizing rabbits with cells from mouse embryos of about 15 days old. The serum was absorbed several times on different normal adult mouse tissues including tissues from the haemopoietic system. When bone marrow rich in X cells was exposed to this serum in cytotoxicity tests, 20% of the nucleated cell population was killed [34]. The serum had an insignificant toxic effect on normal bone marrow controls. Cross absorption on X cell-containing bone marrow inactivated the serum.

Conclusion

The results of the experiments to which reference was made here are concordant in indicating the reappearance in sublethally irradiated adult

mice of an immature, lymphoid cell type, the X cell, present normally in the embryo and the new-born. The observation that the proliferation of X cells is suppressed in the organism by unirradiated adult bone marrow and that, on the other hand, thymectomy enhances proliferation, suggests that X cells are controlled by these organs. The control mechanisms seem to be very radiosensitive. The question whether X cells arise from pre-existing ancestral remnant cells, or whether they are particular descendants of pluripotent haematopoietic stem cells, which accumulate when the control mechanism is suppressed by radiation, cannot be answered at present. Available data do not support the hypothesis that X cells represent a transitional maturation step in the B and T system of immunocompetent cells. Therefore, if X cells are the progeny of stem cells, they may be very early precursors for B and T cells or, alternatively, they may be the expression of a developmental step in a lymphoid system different from the immunocompetent system. The latter alternative is conceivable also, if X cells are the progeny of some ancestral cell type and not of pluripotent stem cells. Figure 1 schematically illustrates the possible relationships of X cells to lymphopoiesis. Experiments in progress – which are performed with the aim of developing efficient separation techniques and to test, among other things, the possible role of X cells in the graft-versus host reaction, and the capacity of these cells to produce immunoglobulins – can be expected to give more conclusive data.

In view of the radiation induced accumulation of immature X cells in the bone marrow and thymus, the question arises whether these cells may play a role in radiation leukaemogenesis. The inhibition of both the X cell proliferation and the development of thymic lymphomas in irradiated mice by administration of normal bone marrow, and the enhanced accumulation of X cells in the thymus by repeated irradiations according to a regime which induces lymphomas in a great frequency [35], are suggestive that X cells represent a target for the radiation induced leukaemia virus. Indeed, it has been proposed that an immature cell type in the thymus constitutes such a target [36]. Experiments are in progress to test this possibility by infecting X cells with radioleukaemia virus, and investigate their possible malignant transformation.

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The γ-Chain in a Ghanaian Adult, Homozygous for Hereditary Persistence of Fetal Haemoglobin

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Abstract. Fetal haemoglobin consists of α- and γ-chains. There are two types of human γ-chains, one ($^G\gamma$) in which residue 136 is one of glycine and another ($^A\gamma$) in which that position is occupied by a residue of alanine. Some time ago a homozygote for the high persistent fetal haemoglobin gene was found in Ghana whose sole red cell pigment was fetal haemoglobin. The nature of the fetal haemoglobin in this individual has now been determined. It is entirely of the G-type.

Key Words
Fetal haemoglobin
γ-Chains of Hb F
Haemoglobinopathies
Hereditary persistence of Hb F

An adult Ghanaian homozygous for hereditary persistence of fetal haemoglobin (HPFH) and heterozygous for elliptocytosis whose haemoglobin consisted solely of α- and γ-chains (Hb F) has been described previously [1]. The nature of the γ-chain gene in this patient has now been determined and was found to be of the $^G\gamma$-type.

Materials and Methods

Blood from the patient was sent to the MRC Abnormal Haemoglobin Unit, Cambridge, where standard techniques were used to prepare a 10-percent haemolysate [2]. Globin was prepared from whole haemolysate by the standard

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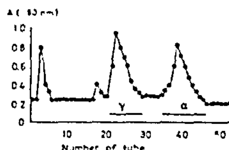


Fig 1 Chain separation of haemolysate of propositus on carboxymethyl cellulose chromatography

HCl acetone precipitation [3] and separated into α and γ chains [4] The chains were recovered after extensive dialysis against 0.5 percent formic acid and freeze drying Fingerprints of the soluble tryptic peptides were made according to SICK *et al* [5] and the insoluble core remaining after tryptic digestion was examined as described by LORKIN *et al* [6] The ratio of ᴳγ versus ᴬγ-chains was determined by amino acid analysis [7] of the γTpXIV peptide after purification by paper electrophoresis at pH 3.5

Results

As can be seen from the elution profile of the globin chains on carboxymethylcellulose (CM) chromatography (fig 1) no other chains were obtained apart from γ¹ and α¹ The complete absence of δ or β-chains confirms that the patient s haemolysate contains only Hb F

The γ-chain was digested with L-(tosylamido 2 phenyl ethyl) chloromethylketone (TPCK) trypsin (Worthington Biochemicals) for 2 h Peptides on diagnostic fingerprints were identified by staining with 0 2 percent ninhydrin as well as by application of platinic iodide (sulphur containing amino acids) Pauly (histidine) Sakaguchi (arginine) 1 nitroso 2-naphthol (tyrosine) and Ehrlich (tryptophan) reagents in various sequences as outlined by EASLEY [8] The staining reactions showed the normal pattern of peptides from ¹ (fig 2) The core was digested with pepsin and fingerprints were made They showed the normal pattern of the peptides on staining with ninhydrin and other reagents for specific amino acids These results confirm earlier observations that this HPFH is not associated with abnormalities in terms of an amino acid substitution



Fig. 2. Fingerprint of soluble tryptic peptides of the γ-chain of propositus.

Table I. Amino acid composition of γTpXIV (γ133-144) of HbF of propositus

Amino acid	Molar ratio	Expected for normal γTpXIV peptide
Threonine	0.98	1
Serine	2.93	3
Glycine	0.91	1 or 0
Alanine	2.17	2 or 3
Valine	2.06	2
Methionine	0.23[a]	1
Leucine	1.07	1
Arginine	0.94	1

[a] Methionine partially destroyed on acid hydrolysis preceding amino acid analysis.

The γTpXIV peptide (residues 133-144) from the preparative fingerprint was purified by paper electrophoresis at pH 3.5, eluted with 6N HCl and hydrolysed under vacuum at 105 °C for 24 h, then subjected to amino acid analysis (table I). There are several possibilities for residue 136 in HbF: Gly or Ala or both of these can be found.

Peptide XIV of the γ-chain												
No	133	134	135	136	137	138	139	140	141	142	143	144
	Met	Val	Thr	Gly or Val	Val	Ala	Ser	Ala	Leu	Ser	Ser	Arg

In the $^G\gamma$-chain 1 Gly and 2 Ala in the $^A\gamma$-chain 3 Ala and no Gly are found If the Hb F comes from a heterozygote various proportions of Gly Ala are present [9]

Discussion

It has been shown by SCHROEDER *et al* [9] that the two γ-chains which exist normally in Hb F differ in position 136 which is occupied by a glycine residue or an alanine residue These γ-chains are products of non allelic genes which appear to be expressed independently Recent studies based on the proportions of γ-chain variants to total amounts of Hb F indicate that there might in fact be four structural genes two for each $^G\gamma$ and $^A\gamma$ [10] The possible arrangements of δ β $^G\gamma$ and $^A\gamma$ genes on the chromosome have been discussed by HUISMAN *et al* [11] and the HPFH condition is thought to arise from a deletion of a whole gene or genes [12] Factors which influence activation suppression or deletion of these genes in HPFH individuals are not yet known and they may not be related to the racial background of these individuals [13] However unrelated HPFH families from India who have so far been studied have been found to synthesise mainly $^G\gamma$-chains [14 15] and SUKUMARAN *et al* [14] believe that the ^{G}-type may be prevalent among the Asiatic Indians This Hb type has also been reported in Negro families with HPFH [14] and all the γβ thalassaemia cases in individuals of Chinese origin [16] On the other hand studies on Greek families with HPFH showed they had $^A\gamma$-chains with a complete suppression of the ^{G}-locus [17] Similar observations have been made in HPFH patients from other Caucasian families [11] Our results indicate that in this homozygote from Accra the $^A\gamma$-locus is non functional

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Homozygous δβ-Thalassaemia

Description of a Case and Review of the Literature

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Abstract. A case of homozygous δβ-thalassaemia is presented with review of the literature. Homozygous δβ-thalassaemia is a haemoglobinopathy where only haemoglobin F is present, haemoglobins A and A_2 being totally absent. Patients with this kind of anomaly usually present with anaemia of intermediate severity. Our case, however, initially presented with severe anaemia associated with jaundice and splenomegaly following an episode of upper respiratory infection. Anaemia subsequently improved without any specific treatment. A pedigree study was also performed.

Key Words
δβ-Thalassaemia
Cooley's anaemia
HbF
Thalassaemia

Homozygous δβ-thalassaemia has been observed in 8 individuals up to this date, representing the Italian [1, 21], Greek [4] and Arabic ethnic groups [20]. In each of these individuals a moderately severe form of Cooley's anaemia was present and most of them were adults. The possibility of existence of such an anaemia has been suggested since 1961 by Fessas [5]. It is interesting that only HbF is found in homozygous δβ-thalassaemia, while in heterozygous cases HbF is moderately increased and HbA_2 is normal or low. On the other hand the homozygous state causes an anaemia of intermediate severity, whereas the heterozygous state is mostly asymptomatic [1, 10, 18, 20, 23, 25, 27].

Methods

Haemoglobin, haematocrit (PCV) and red cell measurements (RBC) were performed by the [illegible] methods and [illegible]

methods respectively Reticulocyte count bone marrow examination serum iron and bilirubin levels were also performed Thin blood films stained with May Grunwald Giemsa stains were inspected for red cell morphology Osmotic fragility and sickling tests, as well as special stains for inclusion bodies in the erythrocytes [7] were made

Blood was collected in vials containing heparine and washed 3 times in 0.85% saline The washed packed cells were lysed by the addition of an equal volume of distilled water and the stroma removed by centrifugation using toluene The haemoglobin concentration was adjusted to 10 g/100 ml by adding distilled water

Determination of HbF was made by the method of SINGER *et al* as modified by FISHER [9] and by electrophoresis in acid agar pH 5.9 [10] Haemoglobin electrophoresis was also performed in cellulose acetate as described by KOHN [14] in starch gel in pH 8.6, in starch block for the elution of HbA_2 and in paper Special stain of HbF in red cells was also made according to KLEIHAUER *et al* [11] DEAE cellulose chromatography was not technically successful

Case Report

A 22 year-old male patient and his family have been examined clinically and haematologically The propositus a rather well developed male was in satisfactory state of health until March 1972 At that time he had an upper respiratory infection which lasted 8 days Following recovery he complained of palpitations and fatigue He was admitted to the 424 General Military Hospital of Thessaloniki on May 5 1972, for evaluation and treatment Anaemia slight jaundice moderately mongoloid facies with epicanthus, palpable liver and spleen were the main clinical features The spleen exceeded the left costal margin by 6 cm

Laboratory examinations PCV 16% Hb 4.2 g% RBC 1.8×10^6 mm^3 MCH 23 pg MCV 88 μm^3 MCHC 25% WBC 6000 mm^3 Red cell morphology was characterized by hypochromia microcytosis poikilocytosis schistocytosis, anisocytosis and numerous target cells The serum iron level was 157 μg% and reticulocyte count 8% In bone marrow smears a significant hyperplasia particularly of the red cell precursors was evident as well as obvious haemosiderin deposits in the erythroblasts and the reticular cells

Electrophoretically only HbF was detected while HbA and HbA_2 were totally absent The fetal haemoglobin measured as 87% by the 1 min method was ultimately determined as 100% by electrophoresis on agar gel at pH 5.9 [10] The blood picture of the patient gradually improved without any treatment He was discharged 2 months later in satisfactory condition (PCV 31% Hb 9 g%) Radiographically a mosaic appearance of the lower end of the humerous due to osteoporosis was demonstrated

The patient's parents and his brother (fig 1) had no clinical signs and symptoms although blood examination revealed deranged red cell morphology moderately increased HbF (4–5.8%) and normal or low levels of HbA_2 (2 2.8%) (fig 2) Their laboratory features are summarized in table 1 They all originated from Kavala a town of northern Greece

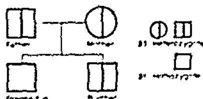


Fig. 1. Pedigree containing β heterozygotes and the β homozygote

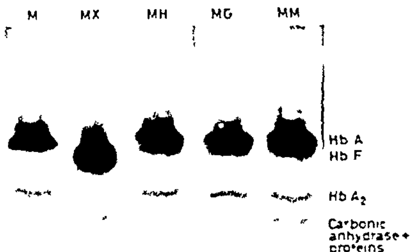


Fig. 2. Haemoglobin electrophoresis of the family in cellulose acetate. From left to right: normal (M), patient (MX), brother (MH), father (MG) and mother (MM).

Discussion

There are various types of thalassaemia each caused by different abnormal genes which suppress either totally or partially the synthesis of the particular peptide chains of haemoglobin [2, 16, 19, 26]. In α and β thalassaemia the synthesis of α and β chains is respectively suppressed. The A_2 and F thalassaemia referred by Fessas [9] and Zoutaris *et al.* [26] belong according to the classification of Ingram and Stretton [12]

[15, 17] It also appears that $\beta\delta$-homozygotes compensate the suppressed β- and δ-chain synthesis more effectively than β thalassaemia homozygotes by synthesizing more γ-chains

In conclusion it is evident that in $\beta\delta$-thalassaemia the activity of β- and δ genes is completely suppressed causing anaemia of the intermediate severity in the homozygous state

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Binding of Folic Acid to Serum Proteins

III. The Effect of Pernicious Anaemia

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Abstract. In untreated pernicious anaemia, the binding of folic acid activity (FAA) to protein zone II of the serum (transferrin) had increased distinctly and to zone I (γ-globulin) slightly. A decrease on the other hand was noted in zones IV–V (α_2-macroglobulin and albumin). During specific treatment the changes return to normal with FAA diminishing in zone II and increasing in zones IV–V. There were still some changes present after 7 weeks of treatment, when the anaemia had already been cured. In a pernicious anaemia patient with neuropathy the chromatogram was not much different from normal and a slight increase in FAA was noted during treatment in protein zones I, II and IV.

Key Words
Carrier proteins of folic acid
Folic acid metabolism
Gel chromatography
Pernicious anaemia
Serum proteins

In vitro, about half the total folic acid activity (FAA) of the serum is bound to proteins [7, 8]. It has earlier been ascertained in our laboratory that this bound fraction was eluted from the chromatography column in 3 different maxima [7]. The FAA carrier proteins of these maxima were found to be the α_2-macroglobulin, transferrin and albumin [7, 10] in this order of magnitude. The FAA maxima are quantitatively very similar in healthy humans, although the FAA of the transferrin zone may be somewhat higher in women than in men [13]. The same person in identical experimental conditions gave an almost exactly identical FAA chromatography [13].

Diphenylhydantoin increases the transferrin binding of FAA [12]. Liver diseases, myeloma and hypothyroidism were also found to play a part [12]. Pronounced changes take place in the protein binding of FAA during pregnancy [11].

It has long been known that disorders in FAA metabolism apparently accompany pernicious anaemia. Sometimes the FAA is considerably higher than normal and at other times at a normal level [19-21]. FAA has some causal connections, unascertained in detail, with the neurological symptoms of pernicious anaemia [17]. A preliminary report on the changes of this binding in pernicious anaemia is presented.

Materials and Methods

The preliminary series comprised 7 patients with pernicious anaemia; their detailed characteristics are given in table I. Because of their anaemia they were all admitted for examination under clinical conditions and none showed any concurrent disease unrelated to pernicious anaemia. None had received specific treatment before the basic examination. The cases concerned were typical instances of pure pernicious anaemia.

Determination of the type of anaemia was based on a thorough examination of peripheral blood, a bone marrow specimen, Schilling's test, ascertainment of achlorhydria, examination of serum iron, FAA and vitamin B_{12}, and experimental

Table I. Haematological data in patients with pernicious anaemia

Case No	Age years	Sex		A	B	C
1	62	w	Hb g%	8.8	–	13.0
			Leuc./mm³	3 500	–	6 000
2	70	m	Hb g%	8.6	–	13.6
			Leuc./mm³	3 200	–	6 300
3	50	m	Hb g%	6.7	8.2	13.7
			Leuc./mm³	3 700	5 800	6 000
4	70	m	Hb g%	8.9	10.0	14.2
			Leuc./mm³	5 100	5 000	7,200
5	67	w	Hb g%	10.9	12.0	13.[illegible]
			Leuc./mm³	6 000	6 100	6 400
6	51	m	Hb g%	8.2	13.0	14.0
			Leuc./mm³	3 600	5 000	6,100
7	69	m	Hb g%	10.2	11.6	13.8
			Leuc./mm³	4 800	5 600	5 900

A = Before treatment; B = after 2 weeks of treatment; C = after 7 weeks of treatment.



Fig. 1. DEAE Sephadex A 50 chromatogram of the serum of a healthy individual. Protein areas marked I–V, vertical lines = FAA × 100 pg/ml.

specific B_{12} therapy. All patients underwent a neurologic examination, but definite signs of neuritis in the limbs could be shown only in patient No. 7 (table I). Roentgenologic examination of the stomach was also carried out in all cases, to exclude the possibility of cancer. The control series consisted of 9 healthy individuals.

The first chromatographic sample (sample A) was taken after the primary clinical examination (before Schilling's test) and before any treatment of the anaemia had been started. After this, therapy was instituted with injections of vitamin B_{12}, 1,000 µg given twice weekly, under clinical conditions. The second chromatography sample (sample B) was taken after 2 weeks of treatment, and the third (sample C) after 7 weeks of treatment (table I, which shows the rise of haemoglobin during therapy). With the third sample, the anaemia was considered to have been completely cured.

The chromatography samples were taken from venous blood and immediately treated in laboratory conditions, starting the DEAE Sephadex A 50 chromatography studies as described in detail earlier [9]. The FAA of the serum and fractions, and the proteins, were determined as described previously [8].

Results

The results of the chromatography studies are presented in tables II and III. Figure 1 shows the chromatogram of a healthy individual, figure 2 shows chromatograms during treatment in a patient without neuritis, and figure 3 shows chromatograms during treatment in a patient with definite neuritis of the limbs. The protein areas in the chromatographies (fig. 1) are indicated by I–V. The numerical chromatography results of the patient with neuritis are given in table IV.

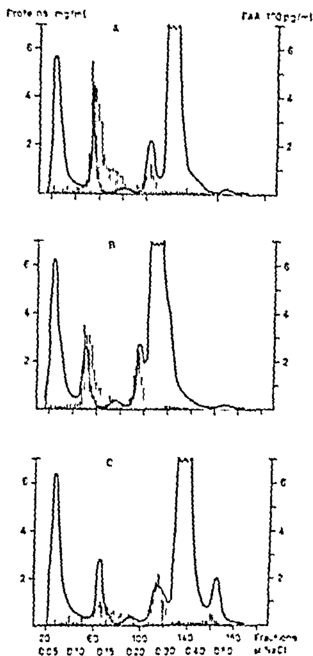


Fig 2 Chromatograms in a pernicious anaemia patient without neuritis A = Before treatment B = after 2 weeks of treatment C = after 7 weeks of treatment

Table II. Mean values of the chromatograms in 6 patients (cases 1–6, table I) with pernicious anaemia before treatment

Protein zones	Pernicious anaemia patients, mean ± SD		Control subjects, mean ± SD	
Whole serum				
Proteins, mg/ml	64.8	6.6	[illegible]3.3	6.7
FAA, ng/ml	3.8	1.9	4.1	1.3
Bound FAA/prot., ng/g	29.3	6.7	20.6	5.2
Bound FAA, %	45	23	41	19
Zone I				
Proteins, %	14	3	13	3
FAA, %	6	3	3	3
FAA/prot., ng/mg	13	10	4	4
Zone II				
Proteins, %	6	1	7	1
FAA, %	49	23	21	8
FAA/prot., ng/mg	235	121	63	45
Zone III				
Proteins, %	1	0.4	2	1
FAA, %	13	12	17	3
FAA/prot., ng/mg	378	575	121	76
Zone IV				
Proteins, %	7	1	7	2
FAA, %	18	10	41	7
FAA/prot., ng/mg	76	31	122	35
Zone V				
Proteins, %	72	5	70	3
FAA, %	14	11	22	11
FAA/prot., ng/mg	5	4	6	3

Zones I–V refer to protein zones shown in figure 1. Average chromatographic results of 9 healthy control subjects.

In untreated pernicious anaemia (table II) a pronounced increase in FAA is seen in protein zone II and a slight increase in binding in zone I. A decrease, compared with the normal, has taken place in zones IV–V. Owing to the wide range, the change in zone III is indistinct.

During specific treatment (table III) the deviations began to move towards normal values. After 2 weeks of treatment (II) FAA is found to

Table III Changes of the chromatograms (mean values) of 4 pernicious anaemia patients (cases 3–6, table I) during treatment

Protein zones	A, mean ± SD		B, mean ± SD		C, mean ± SD	
Whole serum						
Proteins, mg/ml	67.5	7.6	66.8	9.8	72.2	6.2
FAA, ng/ml	4.1	1.9	3.2	0.4	2.8	0.7
Bound FAA/prot., ng/g	28.7	6.8	31.3	10.8	22.0	4.7
Bound FAA, %	49.0	17.0	64.0	26.0	60.0	22.0
Zone I						
Proteins, %	14	3	14	4	16	4
FAA, %	4	1	5	2	5	3
FAA/prot., pg/mg	9	5	14	12	7	6
Zone II						
Proteins, %	5	1	6	1	7	0.5
FAA, %	51	26	39	15	33	8
FAA/prot., pg/mg	264	144	179	79	106	46
Zone III						
Proteins, %	1	0.5	1	0.6	1	0.5
FAA, %	10	4	20	12	17	9
FAA/prot., pg/mg	206	122	288	162	186	96
Zone IV						
Proteins, %	7	0.5	7	0.6	7	0.8
FAA, %	22	12	25	4	31	8
FAA/prot., pg/mg	82	29	104	29	94	15
Zone V						
Proteins, %	72	3	71	5	69	5
FAA, %	6	2	12	6	14	8
FAA/prot., pg/mg	2	1	5	2	5	2

A = Untreated, B = after 2 weeks of treatment, C = after 7 weeks of treatment. Protein zones as in figure 1.

decline in protein zone II. The decline continued after 7 weeks of treatment (C) and the mean normal values were not yet reached (table II, controls), although the anaemia was cured. An increase in FAA binding took place throughout the period of observation in protein zones IV–V, in neither were the normal mean values reached within the 7-week treatment. No definite conclusions can be drawn from the FAA changes in protein zone III.

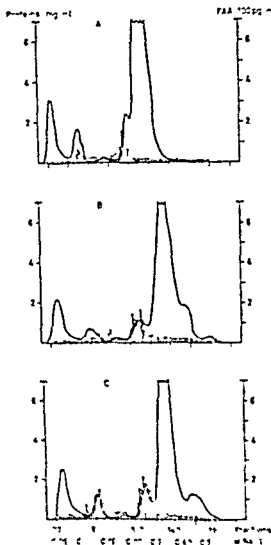


Fig. [illegible] Chromatograms of the pernicious anaemia patient [illegible] The [illegible]

Table IV. Values of the chromatograms (fig. 3) of the pernicious anaemia patient with neuritis (case 7, table I)

Protein zones	A	B	C
Whole serum			
Proteins, mg/ml	64.4	59.6	62.0
FAA, ng/ml	18.4	5.0	4.8
Bound FAA/prot., ng/g	14.9	20.4	29.1
Bound FAA, %	5.0	23.0	39.0
Zone I			
Proteins, %	12	13	13
FAA, %	1	5	7
FAA/prot., pg/mg	1	8	15
Zone II			
Proteins, %	6	4	5
FAA, %	23	13	30
FAA/prot., pg/mg	52	66	185
Zone III			
Proteins, %	1	1	1
FAA, %	15	11	10
FAA/prot., pg/mg	208	170	246
Zone IV			
Proteins, %	6	7	8
FAA, %	37	41	37
FAA/prot., pg/mg	99	116	132
Zone V			
Proteins, %	75	75	73
FAA, %	25	30	17
FAA/prot., pg/mg	5	8	7

A = Untreated, B = after 2 weeks of treatment, C = after 7 weeks of treatment. Protein zones as in figure 1.

In the patient with pernicious anaemia and neurologic symptoms (table IV) a higher than normal total serum FAA was recorded but it was normalized during treatment. The percentage of binding to serum proteins increased accordingly. The binding of FAA to protein zone I increased, as did binding per unit of protein in zone II. A parallel trend is seen also in zone IV. Zones III and V are without changes. Taken as a whole, the chromatogram is very close to normal, and the changes dur-

ing therapy are unlike those found in the other pernicious anaemia patients, being very slight and having a different trend

Discussion

The present study shows that untreated pernicious anaemia involves changes in the FAA binding ratios compared with those in a healthy individual. In a patient with neuropathy changes of this type were not noted.

Folic acid metabolism in pernicious anaemia has long attracted the attention of research workers. Both vitamin B_{12} and folic acid participate in the biosynthesis of methionine, also in humans, by transferring the methyl group to homocysteine [16]. It has been experimentally ascertained that massive folic acid doses can correct pernicious anaemia transiently whereas minor doses have practically no effect [3, 4, 14]. Administration of folic acid can aggravate the neurologic symptoms of pernicious anaemia [17]. Furthermore, it has been observed that the tissue uptake of a parenteral dose of folic acid, as measured by the plasma clearance [1] or urinary excretion [18], is increased in patients with pernicious anaemia, suggesting tissue depletion of folic acid. After a histidine loading test, the pernicious anaemia patient excretes more formiminoglutamic acid than normal into the urine [5], another observation giving much the same purport. On the other hand, it has been possible to show that the serum FAA of the pernicious anaemia patient can be completely normal or sometimes very high [19-21]. Chanarin and McLean [2] have referred to a possible causal connection between the neurologic symptoms and high serum FAA.

The close relationship which is apparent in haematopoiesis between vitamin B_{12} and folic acid appears to be the result of some role of cobalamins in the utilization of folates, but this has yet to be clearly defined [15]. A hypothetic obstacle for the use of folic acid mentioned in the literature is the methyltetrahydrofolate trap [6]. The clinical effects of cobalamin deficiency are consistent with the trapping of folates as methyltetrahydrofolic acid and the subsequent intracellular operational deficiency of other folate coenzymes [15].

Research into the proportional distribution of the FAA bound to the respective serum proteins is of recent origin. It is striking to note that protein zone II in the chromatographies reveals special activ

ity in the metabolic events of folates. We have noted this during pregnancy [11], in liver diseases and hyperthyroidism, and under the influence of diphenylhydantoin [12]. Usually, when the FAA of protein zone II increases, that of zones IV and V decreases, and vice versa. The same is now seen very clearly in pernicious anaemia. It may perhaps be concluded that, in pernicious anaemia, changed ratios of FAA binding are rather an indication of disorders in folate metabolism than a change in total serum FAA. So far, the cause of such a change in FAA binding in pernicious anaemia and its possible role as transmitter of metabolic interaction between vitamin B_{12} and FAA, remains purely hypothetical. The observation that the pernicious anaemia patient with neuropathy had a chromatogram very different from that of the others, and that the changes in binding were few and slight during therapy, is of particular interest. The findings of the present study, as far as we know, are new, and following these preliminary results an extensive material is being collected. Special attention will be paid to the neurologic symptoms of pernicious anaemia and to their causal connection with the problems of FAA binding.

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Folate Binding in Animal Plasma[1]

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Abstract. Plasma from pig, sheep, goat, cattle, horse, rabbit, dog, rat, guinea pig, and chicken was examined for the presence of specific binders for folic acid. It was found that only pig plasma contains unsaturated and specific binders of folic acid. The maximum binding capacity of pig plasma for folic acid was found to be 14.0–26.5 ng/ml of plasma. These binders have a lower affinity for other folates and folate analogues. They are partly saturated by endogenous folates and they move electrophoretically in veronal buffer, pH 8.6, in an area including β-globulin and most of the γ-globulin fraction. Specific binders of folic acid with high binding capacity were also found in one female sheep out of the 16 tested.

Key Words
Carrier proteins of folic acid
Folate binding
Folic acid in animals
Pig plasma
Plasma folic acid

We recently reported that plasma from large pigs contains specific binders of folic acid (FA) which react also with tetrahydrofolic acid and *N5*-methyltetrahydrofolic acid (MTFA) [5]. Plasma taken from these animals has been successfully used, as the source of binders, for the determination of human serum folates in a newly developed assay, based on the principles of saturation analysis [5]. The plasma of a small number of other animals, such as sheep, goats, cattle and rats, was not found to have specific binders either for ^{3}HFA or ^{14}CMTFA [5]. The present report describes experiments performed to examine a large group of various species of animals, for the presence of folate binders in their plasma. Human plasma was also examined under the same experimental con-

[1] Supported by a grant from the International Atomic Energy Agency (contract No. 1066/R1/RB).

ditions Furthermore, certain properties of the binders found in pig plasma were investigated

Materials and Methods

Heparinized plasma from animals of 10 different species and man was studied. At least 4 animals from each species were used. ³H FA of high specific activity (15-40 Ci/mmol) was obtained from Amersham Searle, Inc. The radiochemical purity was tested by paper chromatography in butanol pyridine water (1 1 1), and only material with radiochemical purity higher than 9[illegible] % was used.

To test the ability of each plasma to bind FA, the following procedure was followed in duplicate. 1.5 ml of 0.2 M phosphate buffer, pH 7.4, 1 ng of ³H FA, 0.25 ml or 0.025 ml of animal plasma, and water to ensure a final volume of 2 ml were added and mixed in a tube. The mixture was incubated at room temperature for 1 h. Then 1 ml of a suspension of 0.25% charcoal in 0.15 M phosphate buffer, pH 7.4 containing 0.025% dextran of average molecular weight 70 000 was added, and the tubes were shaken for 5 min. After centrifugation, 2.5 ml of the supernatant fluid were added to 6 ml of Insta Gel (Packard Instrument Company) and counted.

A supernatant control, to measure nonadsorbable radioactivity, was prepared as described for the test sample, but without plasma. The counts of this control which were less than [illegible] % of the total radioactivity, were subtracted from the counts of each sample. Correction for quenching effect was also made.

The dilution effect of cold FA on the degree of binding of ³H FA by the various plasmas was studied by adding 1 ng of cold FA to ³H FA before incubation with the plasma.

The maximum binding capacity of pig plasma for FA was determined by adding increasing amounts of ³H FA to a fixed quantity of plasma and after the usual treatment, the amount of bound ³H FA was measured.

To test the specificity of the pig plasma binders, experiments were performed in which increasing amounts of unlabeled FA, or MTHFA, pteroic acid, or N5-formyltetrahydrofolic acid and methotrexate were used separately along with 1 pmol of ³H FA and a quantity of pig plasma to bind about 50% of ³H FA in the absence of other folates. The pig plasma was added after mixing the radioactive and the unlabeled folates or folate analogues.

To study the effect of heating on FA binding, pig plasma, diluted with 4 vol of 0.15 M phosphate buffer, pH 7.4, was heated at various temperatures for 15 min in a water bath, cooled and tested for binding activity.

Cellulose polyacetate strip electrophoresis, in veronal buffer, pH 8.6, was performed on pig plasma incubated with ³H FA or without tracer. After electrophoresis, the strip was cut in 2 parts, one of which was stained with bromophenol blue. The unstained part was cut according to the stained sample, and eluted before drying with 1 ml of 0.15 M phosphate buffer, pH 7.4. When pig plasma containing ³H FA was used, the eluate was added to 10 ml of Insta Gel and counted. The eluates from the samples which did not contain tracer, were tested for protein binding [illegible] after [illegible] ³H FA.

Results

Table I shows the percentage of bound radioactivity of ^{3}HFA by the plasma of several species. It can be seen that under the present experimental conditions 0.025 ml of pig plasma bound 30.5–65.4% of the added radioactivity. A tenfold higher quantity (0.25 ml) of the plasma from other animals bound less than 7%, with the exception of horse plasma (0.25 ml of horse plasma from 5 animals bound 7.2–10.3% of the radioactivity). Furthermore, there was one striking exception: 1 µl of plasma from a female sheep bound 45% of the ^{3}HFA radioactivity. Unfortunately, the blood of this sheep was obtained from a slaughter house, so there is no other information about that animal except that it was more than 2 years old. 0.25 ml of human plasma, tested in the same way, bound 2.1–7.8% of the added radioactivity.

The addition of the cold FA to ^{3}HFA before incubation with plasma reduced the amount of bound ^{3}HFA only in the case of pig plasma and the plasma of the sheep with the high binding capacity. In these cases a decrease of more than 40% of the bound ^{3}HFA was measured.

The maximum binding capacity of plasma samples from 9 different

Table I. Binding of 1 ng ^{3}HFA by plasma of several species

Species	Number of experiments	Quantity of plasma ml	Bound fraction range[1]
Pig	18	0.025	30.5–65.4
Sheep	15	0.250	2.1–4.8
Sheep[2]	1	0.001	45.0
Goat	4	0.250	2.0–3.2
Cattle	4	0.250	2.8–4.6
Horse	5	0.250	7.2–10.3
Rabbit	4	0.250	3.0–4.2
Dog	4	0.250	2.7–6.8
Rat	4	0.250	5.4–6.9
Guinea pig	4	0.250	3.1–4.2
Chicken	4	0.250	3.2–5.7
Man	20	0.250	2.1–7.8

[1] Expressed as percentage of bound to the total added radioactivity.
[2] See text.

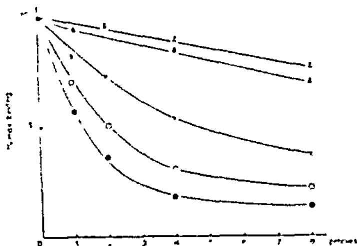


Fig. 1. Effect of increasing concentrations of various folates on the amount of 1 pmol of ³HFA bound by pig plasma. Binding is reported in percent of the maximum binding obtained in the absence of unlabeled folates. ● = FA, ○ = MTFA, × = pteroic acid, ▲ = 5-formyltetrahydrofolic acid, and △ = methotrexate.

male and female pigs, aged between 2 days and 3 years ranged between 14.0 and 26.5 ng/ml of plasma.

Figure 1 shows the effect of increasing concentrations of various folates and folate analogues on the quantity of ³HFA bound by pig plasma. It is evident that all tested substances possess displacement activity for ³HFA and that FA has the highest binding affinity for pig plasma. A quantity of L-glutamic acid 10,000 times higher than that of ³HFA had no effect on the amount of ³HFA bound by pig plasma.

Heating of the pig plasma for 10 min had the following effects on FA binding: at 80 °C, 15% of activity was lost and almost all at 90 °C, while at 70 °C no loss of activity was observed. A pH range between 6.5 and 8.5 did not substantially influence the binding capacity of pig plasma. After electrophoresis, in both the pig plasma incubated with ³HFA and that without tracer, binding activity was found in an area including β globulin and most of the α-globulin fraction.

To investigate whether pig plasma binders are partly saturated with endogenous folates, extracts of plasma from 5 animals were prepared by boiling for 10 min each plasma sample with 4 vol of 0.15 M phosphate

buffer, pH 7.4, in the presence of 1% ascorbic acid. A volume of 0.25 ml of each extract was added to the usual incubation mixture, containing 1 ng ^{3}HFA and a quantity of pig plasma to bind about 50% of ^{3}HFA. The addition of the extracts reduced the bound fraction of ^{3}HFA by 24–42%, suggesting that pig plasma binders are indeed partly saturated with folates.

Discussion

Our results show that among a number of tested plasma samples from 10 different species, only pig plasma contains unsaturated specific binders with high affinity for folic acid. The other animal plasma samples have a very small binding capacity for ^{3}HFA (similar to the human plasma) with the exception of one female sheep, in which a very high concentration of unsaturated specific binders for FA was found. This small binding capacity of animal plasma was not affected by the addition of cold FA. This probably indicates that specific folate binders are not present in their plasma, and that the small binding capacity found is due to nonspecific binders. Another explanation can be that specific folate binders in these plasmas have a low affinity for folic acid, and thus ^{3}HFA under the present experimental conditions is adsorbed by the coated charcoal. This does not seem very probable since similar results were obtained for 5 species in a previous work [5] in which the separation of bound from unbound radioactivity was performed under mild conditions using Sephadex G-25 microcolumn. The possibility that specific folate binders with high affinity are saturated by an excess of folates cannot be excluded. In any case, pig plasma, when compared to the plasma of a number of domestic and experimental animals and man, is unique in the sense that it only contains unsaturated specific folate binders whose affinity for FA and MTFA is high enough to permit the development of an assay for human plasma, based on the principles of saturation analysis [5].

Several methods have been employed to examine human plasma for the presence of folate binders in experiments performed with ^{3}HFA. The separation of bound from the unbound form was achieved with coated charcoal [10], ultrafiltration [1, 4] and equilibrium dialysis [4]. These procedures have shown the existence of weak nonspecific folate binders in human plasma. Similar results have been reported for rat serum by NIAI and WILLIAMS [11] who used ultrafiltration and zonal density gradient electrophoresis for the same purpose. These results are in agreement with

our findings that human and rat plasma do not contain avid unsaturated specific folate binders. Binding of natural endogenous folates of human serum has been also studied. RETIEF and HUSKISSON [12, 13] reported the existence of an nondialyzable folate fraction which was eluted from Sephadex G 25 column in association with serum proteins. Adsorption of serum with hemoglobin-coated charcoal had a folate-clearing effect very similar to that of dialysis. A number of studies by MARKKANEN [6], MARKKANEN and PELTOLA [7] and MARKKANEN *et al.* [8, 9] have revealed that 30–40% of endogenous human serum folates bind to 3 proteins, namely albumin, transferrin and α_2 macroglobulin, the latter being the main folate binder of endogenous serum folates. From these experiments, it cannot be concluded whether any of these 3 proteins is specific for endogenous serum folates. No one of these seems to be similar with the pig plasma specific folate binders of folic acid since the latter, strongly bound with MTHFA, moves on electrophoresis in an area including β-globulin and most of the γ-globulin fraction. The physiologic function of the folate binders of pig plasma cannot be defined at the moment, but it may be related with the mechanism of folate uptake by the tissues.

The finding that the plasma of only one sheep among 16 had a high binding capacity for folates suggests that some abnormality in sheep may result in the release of specific folate binders into the blood stream.

Specific binders, with binding capacity for several substances of biological importance, have been used for the development of assays for their determination based on the principles of saturation analysis. The present studies have shown that pig plasma binders have different affinities for various folates. Similar results were obtained by others for specific folate binders found in milk [2, 10]. Therefore, in order to apply saturation analysis for folate determination in various biological materials, using pig plasma or milk as a source of folate binders, it is necessary to know the exact nature of these compounds which constitute a group of substances with structural variations. The main fraction in human serum has been identified as MTHFA [3] and so this substance has been used for the preparation of the standard curve in saturation analysis methods, using folate binders of pig plasma [5] and milk [14, 15].

The finding that an extract of pig plasma possesses displacement activity for MTHFA (which indicates that pig plasma binders are partly saturated by folates reacting with these binders) suggests that other animals may also contain similar folates which, therefore, could be determined with the aid of pig plasma binders.

buffer, pH 7.4, in the presence of 1% ascorbic acid. A volume of 0.25 ml of each extract was added to the usual incubation mixture, containing 1 ng ³HFA and a quantity of pig plasma to bind about 50% of ³HFA. The addition of the extracts reduced the bound fraction of ³HFA by 24–42%, suggesting that pig plasma binders are indeed partly saturated with folates.

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The Relevance of Immune Reactions in Acute Favism[1]

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Abstract. In fava bean extracts the proteins were separated and characterized by gel filtration on Sephadex G-25 columns, cellogel electrophoresis and analytical ultracentrifugation. The dried extract and its chromatographic fractions were used to detect serum antibodies to fava beans by passive haemagglutination tests. 75 patients affected by acute favism were studied by this method. Haemagglutination tests were positive in 5 patients (6.6%) at the onset of the haemolytic crisis but a rapid fall of the titre was observed in the subsequent days. β_{1C}/β_{1A} and immunoglobulin levels showed no significant changes. Our results do not support the view that hypersensitivity to fava bean may be involved in the haemolysis of acute favism.

Key Words
Antibodies in favism
Fava bean extract
Favism
Gel filtration

The pathogenesis of acute favism, a haemolytic syndrome which occurs in some G-6-PD deficient subjects following the ingestion of fava beans or the inhalation of their pollen, has not been completely established. *In vitro* experiments have shown that fava bean extracts oxidize reduced glutathione of G-6-PD deficient red cells after a short period of incubation [1, 2]. The oxidation seems to be carried out by a low molecular weight fraction of the fava bean extract [3]. On the other hand, some clinical evidence, as the lack of relation between the amount of fava beans ingested by sensitive subjects and the occurrence of the haemolytic crises, suggests that other factors may be involved in acute favism. The existence of an abnormal gene inducing a familial predisposition to favism has been demonstrated in the Greek population [4]. Roth and Frumin [5] have reported that a serum factor is deficient in these patients. The direct

1 Supported in part by funds from the CNR, contract No. [illegible]

The discovery of new binders, which would differ in specificity from those of pig plasma and cow milk, would help in the study of folates existing in the biological material of humans or animals. The finding of specific binders for folic acid in a female sheep shows that more extensive studies for the search of folate binders would help in this direction.

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The pathogenesis of acute favism, a haemolytic syndrome which occurs in some G-6-PD deficient subjects following the ingestion of fava beans or the inhalation of their pollen, has not been completely established. *In vitro* experiments have shown that fava bean extracts oxidize reduced glutathione of G-6-PD deficient red cells after a short period of incubation [1, 2]. The oxidation seems to be carried out by a low molecular weight fraction of the fava bean extract [3]. On the other hand, some clinical evidence, as the lack of relation between the amount of fava beans ingested by sensitive subjects and the occurrence of the haemolytic crises, suggests that other factors may be involved in acute favism. The existence of an autosomal gene inducing a familial predisposition to favism has been demonstrated in the Greek population [4]. Roth and Frumin [5] have reported that a serum factor is deficient in these patients. The defect

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has been more recently identified by NATHAN *et al* [6] as a qualitative IgA deficiency

Several investigations moreover have put forward some evidence that an immunologic mechanism operates in this condition KANTOR and ARBESMAN [7] and KANTOR *et al* [8] have demonstrated antibodies specific to fava beans in the serum of 2 patients, while MARCOLONGO [9] found atypical antibodies in the patient's sera against their own red blood cells

The purpose of this investigation is to establish in a large series of sensitive subjects the incidence of serum antibodies to fava beans Some physio-chemical properties of the proteins contained in the fresh and dried fava bean extracts employed for the haemagglutination tests have also been investigated

Material and Methods

Patients Serum samples were taken during acute favism in 75 Sardinian subjects who were admitted to Cagliari University Hospital during 2 successive years Samples were taken on admission and on the 4th and 7th day In 15 patients control tests were carried out 3 months after the crisis. None of the patients received blood transfusions Sera from laboratory staff were used as normal controls all the controls were fava bean eaters All serum samples were kept frozen at –30 °C until used

Fava bean extracts Dried fava bean extracts were prepared as described by KANTOR et al [8] Dried fava beans, reduced to a fine powder, were defatted in a Soxhlet apparatus A 5 percent suspension in phosphate buffered saline pH 7 4 was made up, centrifuged at 2,500 g for 20 min and the sediment was discarded

Fresh fava bean extracts were prepared using the method of Walker and Bowman with minor modifications [11] Fresh young beans (8 14 mm maximum diameter) were soaked in a large quantity of water at 5 C After 12 18 h water was drained off and the beans were finely ground in phosphate buffered saline pH 7.2 in a Waring blendor at 4 °C (2 volumes of saline were added to 1 volume of beans) The suspension was filtered through several layers of gauze and centrifuged at 37 000 g at 0 °C in a Sorvall RC-2 refrigerated centrifuge The sedimented fraction was discarded and the clear supernatant fluid was kept frozen until required

Gel filtration Fresh and dried fava bean extracts were submitted to gel filtration through a Sephadex G 25 (200–400 mesh) column Samples of 10 ml were applied and eluted with 0 01 M tris HCl buffer pH 7.5 The effluent was monitored at 253 nm by a LKB Uvicord Ultraviolet absorptiometer and collected in 5 ml fractions. These fractions were pooled according to the appearance of the absorption peaks and then lyophylized Before the performance of the tests the pooled fractions were reconstituted to 10 ml with buffered saline pH 7 4 For electrophoresis the lyophylized material was reconstituted with smaller volume of diluent

Electrophoresis was carried out on cellogel strips (17.5 × 10 cm). The following buffers were used: 0.05 M barbital pH 8.6; 0.05 M phosphate pH 7.5; tris-EDTA-citrate pH 7.5 (0.075 M tris, 0.004 M EDTA, 0.013 M citric acid). A constant voltage of 8 V/cm for 3 h was applied.

Ultracentrifugation analyses were carried out in a Spinco Model E apparatus at 52,640 rpm.

Haemagglutination test. Boyden's method modified according to Kastrom and Anertman [7] was applied. The dried fava bean extract or its chromatographic fractions were absorbed on tanned sheep red cells and then incubated with serial dilutions of subject serum heated at 56 °C for 30 min. Haemagglutination titres are reported as the reciprocal of the maximum dilutions yielding positive reactions.

For each series of tests controls with normal human rabbit serum and with the antiserum to fava beans were performed. Antiserum to the whole fava bean extract was prepared in the rabbit according to Kastrom and Anertman [7].

Immunochemical quantitation of serum β_{1C}-β_{1A} globulin and immunoglobulins (IgG, IgA, IgM) were carried out by single radial immunodiffusion on cellulose acetate strips according to Vincent *et al.* [10]. Specific antisera (Behringwerke, Marburg/Lahn) were diluted with Veronal buffer (0.1, pH 8.6) in the following proportions: IgG 1:9, IgA 1:6, IgM 1:20, β_{1C}-β_{1A} 1:18. Calibration curves were obtained using a standard human serum with a known concentration of the proteins under investigation (Standard Human Serum, Behringwerke Op. No. 265).

Results

Characterization of the fava bean extract. In the elution patterns on Sephadex G-25 columns of fresh and dried fava bean extracts as monitored at 253 nm, proteins were present in the first peak only – their appearance and characteristics were constant in different preparations. The pattern of the subsequent fractions was rather constant for the dried fava bean extract but showed some variability in different preparations of the fresh fava bean extract.

The electrophoretic pattern of both fresh and dried fava bean extracts is shown in figure 1. Two zones are detectable with a mobility similar to that of α_1 and β globulins.

Analytical ultracentrifugation of the proteins of the dried fava bean extract showed 3 different components with a constant of 4, 7 and 10 S, respectively. The 7 S component was predominant.

Haemagglutination test. Only 5 patients showed positive haemagglutination tests on the first day. The titres are reported in table I; the highest was 2,048.

Sera from normal subjects as well as normal rabbit serum gave no ag-

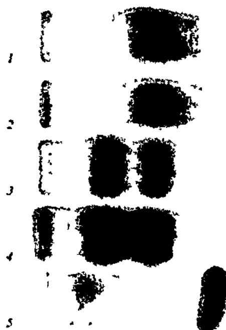


Fig. 1. Electrophoretic pattern of fava bean extracts. 1–2, whole fava bean extract; 3–4, chromatographic fractions (1 and 2 of dried fava bean extract); 5, normal human serum. 0.05 M barbital buffer pH 8.6.

Table I. Haemagglutinating titres of the sera of 5 patients at the onset of the haemolytic crisis

Patients	Haemagglutinating titres
A. B.	2,048
M. G.	512
B. E.	16
P. L.	128
M. L.	64
Antifava rabbit serum	4 096

Titres are reported as the reciprocal of the maximum dilutions yielding positive reactions.

Table II Haemagglutinating titres of patient A. B.'s serum obtained using single chromatographic fractions or fava bean whole extract

	Whole extract	Chromatographic fractions of fava bean extract					
		1	2	3	4	5	6
Patient A. B. serum							
Haemagglutinating titre	2,048	128	32	0	0	0	0
Anti fava rabbit serum							
Haemagglutinating titre	4,096	2,048	64	0	0	0	0

Table III Serum levels (mg/100 ml) of β_{1c}/β_{1a} globulin and immunoglobulins in patients with acute favism and in normal subjects (mean ± SE)

	75 patients with acute favism	40 normal subjects
β_{1c}/β_{1a}	83.0 ± 3.90	87.5 ± 2.44
IgG	1,226 ± 44.2	1,225 ± 23.8
IgA	173.2 ± 8.13	187.7 ± 7.49
IgM	131.5 ± 6.10	127.7 ± 8.02

glutination at any dilution. A marked decrease of the haemagglutination titre was observed in all the positive sera in the days following the onset of the haemolytic crisis. The serum of patient A. B. which exhibited a titre of 2,048 on the first day was reduced to 128 on the 7th day; by that time all the others were negative. No positive test was obtained when patients were controlled 3 months after the acute crisis.

Patient A. B.'s serum was tested with the 6 chromatographic fractions of the dried fava bean extract. Positive tests were obtained at high dilution with the first fraction only. Antiserum to fava beans confirmed these results (table II).

Immunochemical quantitation of serum β_{1c}/β_{1a} and Ig. Table III summarizes the mean concentration of β_{1c}/β_{1a} and Ig in 75 subjects with acute favism. The IgG, IgA, IgM serum levels remain within or near the normal range; no changes are apparent in serum levels of β_{1c}/β_{1a} globulin

Discussion

Serum haemagglutinating antibodies against fava beans were reported by Kantor and Arbesman [7] and Kantor *et al.* [8] in only 2 patients with acute favism tested before any blood transfusion was given. They concluded that an immune mechanism such as sensitization to fava beans may be an important factor in the pathogenesis of favism.

Our investigation on a large series of untreated patients shows that only in a small number of patients (6.6%) serum antibodies to fava beans are detectable by Kantor's method. But the relevance of hypersensitivity to fava beans in the pathogenesis of the haemolytic crises could be questioned also for this selected group of patients. Results from several investigators indicate that conditions for an antigen-antibody reaction may occur at the red cell surface in the fava bean eaters. Antibodies to fava beans have been demonstrated in experimental animals fed orally with fava beans without any resulting abnormality in the recipient's red cells [11] and fava bean proteins have been detected adsorbed to human erythrocytes during the haemolytic crisis of favism [12–14]. Nevertheless, the time course variation of serum antibodies observed in our patients is conflicting with the hypothesis of such a reaction taking place at the red cell surface. The highest antibody titers were found just after the onset of the haemolytic crisis, when fixation to red cells carrying the antigen should have reduced their level, while in the following days a rapid fall of the antibody titre was seen. Consumption of the antibody by the antigen is not likely in this period since the recently produced red cells which are predominant in the blood stream at this stage have been shown to be clear of the antigen. A further point against an antigen-antibody reaction as the cause of haemolysis could be the normal serum β_{1C}/β_{1A} level.

Antigenic components of the fava bean extract employed for the haemagglutination tests were eluted with the void volume from a Sephadex G 25 column. Proteins contained in this fraction were shown to consist mainly of 7S by ultracentrifugation analysis as the greatest part of the seed globulins including phytohaemagglutinin [15, 16].

The fava bean extract employed for the haemagglutination tests was the same shown in previous experiments to induce a significant fall of GSH during incubation with G-6-PD deficient cells of Sardinian subjects as reported elsewhere [3]. The components with GSH oxidizing activity were almost entirely contained in the third fraction of the eluate separated by gel filtration [3]. Thus, our *in vitro* experiments support the hypothesis

that a metabolic interaction between G-6 PD deficient red cells and some fava bean components is the most important factor leading to haemolysis, while immunological reactions seem to be 'mere epiphenomena of no pathogenetic importance' [17]

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Discussion

Serum haemagglutinating antibodies against fava beans were reported by KANTOR and ARBESMAN [7] and KANTOR *et al* [8] in only 2 patients with acute favism tested before any blood transfusion was given They concluded that an immune mechanism such as sensitization to fava beans may be an important factor in the pathogenesis of favism

Our investigation on a large series of untreated patients shows that only in a small number of patients (6 %) serum antibodies to fava beans are detectable by Kantor s method But the relevance of hypersensitivity to fava beans in the pathogenesis of the haemolytic crises could be questioned also for this selected group of patients Results from several investigators indicate that conditions for an antigen antibody reaction may occur at the red cell surface in the fava bean eaters Antibodies to fava beans have been demonstrated in experimental animals fed orally with fava beans without any resulting abnormality in the recipient s red cells [11] and fava bean proteins have been detected adsorbed to human erythrocytes during the haemolytic crisis of favism [12–4] Nevertheless the time course variation of serum antibodies observed in our patients is conflicting with the hypothesis of such a reaction taking place at the red cell surface The highest antibody titers were found just after the onset of the haemolytic crisis when fixation to red cells carrying the antigen should have reduced their level while in the following days a rapid fall of the antibody titre was seen Consumption of the antibody by the antigen is not likely in this period since the recently produced red cells which are predominant in the blood stream at this stage have been shown to be clear of the antigen A further point against an antigen antibody reaction as the cause of haemolysis could be the normal serum γ_1 β_{1C} level

Antigenic components of the fava bean extract employed for the haemagglutination tests were eluted with the void volume from a Sephadex G 25 column Proteins contained in this fraction were shown to consist mainly of 7S by ultracentrifugation analysis as the greatest part of the seed globulins including phytohaemagglutinin [15 16]

The fava bean extract employed for the haemagglutination tests was the same shown in previous experiments to induce a significant fall of GSH during incubation with G 6 PD deficient cells of Sardinian subjects as reported elsewhere [3] The components with GSH oxidizing activity were almost entirely contained in the third fraction of the eluate separated by gel filtration [3] Thus our *in vitro* experiments support the hypothesis

that a metabolic interaction between G-6 PD deficient red cells and some fava bean components is the most important factor leading to haemolysis, while immunological reactions seem to be 'mere epiphenomena of no pathogenetic importance' [17].

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Turnover of Lysozyme-Positive Monocytes in Normal Rat Blood

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Abstract. Tritiated thymidine was injected into normal rats at 6-hour intervals for 5.5 days, and the labelling of blood monocytes was recorded. Monocytes were identified on the basis of their cytochemical lysozyme activity demonstrated by lysis of bacteria around the cells. No labelled monocytes were found one hour after the first injection, but a few labelled lysozyme-negative mononuclear blood cells were seen. 58% of the monocytes were labelled on the first day and 100% on the fourth day. Both unlabelled and heavily labelled monocytes left the circulation at random with a half-time of 12-13 h. The grain count data suggest that monocytes are the progeny of at least 2 succeeding precursor cells with generation times not exceeding 1.6 days and that part of the monocytes stay in a storage pool before being released into the blood.

Key Words
Autoradiography
DNA synthesis
Lysozyme in blood cells
Monocyte kinetics
Rat blood

Frequent intermittent labelling with tritiated thymidine (³HTdR) provides a relatively simple and reliable method for estimating the turnover time of blood cells in vivo. In an ideal situation every cell entering the blood after the initiation of ³HTdR injections is labelled, and the disappearance rate of the unlabelled cells is a direct measure of the turnover of the blood cell pool.

This principle was first applied to the study of blood monocytes by Whitelaw [?], who injected rats intermittently with ³HTdR and found that unlabelled monocytes left the circulation according to an exponential function with a half-time of 3.1 days. By a continuous labelling method Volkman [6] arrived at a half-time of 4 h for rat blood monocytes. However, later studies using a single injection technique [11] and transfusion of labelled cells [1] resulted in half-times of 13 and 14 h, respec-

tively. A short half-time was also suggested by the observation of SCHAER *et al.* [4] that 100% of rat monocytes were labelled on the fourth day during intermittent injections of ³HTdR.

The notorious problem of reliable morphological identification of rat monocytes was avoided by WHITELAW [9], who used the distinct and reproducible criterion of a positive peroxidase reaction. It was recently shown, however, that a large proportion of the peroxidase-positive mononuclear blood cells in the rat lack the lysozyme activity seen in 96% of the morphologically typical monocytes [7]. This finding suggests that the population of peroxidase-positive mononuclear blood cells in the rat is not homogeneous. It consists of at least 2 types of cells, which can be differentiated cytochemically and may also be kinetically different. In the present study, intermittent injections of ³HTdR were given to rats in order to estimate the turnover time of the lysozyme-positive mononuclear blood cells believed to represent the typical rat monocyte.

Material and Methods

Six male rats of an inbred Wistar strain weighing 90–100 g received an i.p. injection of 0.25 μCi/g of ³H thymidine in 0.5 ml of saline every 6 h. The total number of injections per rat was 22. Each rat thus received a total dose of about 5.5 μCi/g over a period of 5.5 days. The ³H thymidine (specific activity 2 000 mCi/mM) was obtained from the Radiochemical Centre, Amersham, England.

Tail vein blood from each rat and a suspension of dried *Micrococcus lysodeikticus* bacteria were mixed and smeared daily and stained for the demonstration of lysozyme activity as previously described [7]. Half the smears, which were used in the second part of the investigation [6], were stained by the combined method for peroxidase and lysozyme [7]. The stained smears were coated with Kodak NTB2 emulsion and stored for 20 days at 4 °C in light tight boxes containing silicagel as a dehydrating agent. The autoradiograms were developed with Kodak D 19 developer, fixed, and counterstained with Giemsa through the emulsion.

Total leukocyte counts and differential counts of 500 leukocytes were performed on tail vein blood from each rat at intervals of 2–4 days throughout the experiment.

Definition of monocytes. Monocytes were identified as mononuclear leukocytes surrounded by a broad zone of lysed bacteria. As previously shown [7] this sign of lysozyme activity differentiates rat blood monocytes from lymphocytes. Simultaneous staining for peroxidase was not considered necessary since nearly all lysozyme positive mononuclear blood cells in the rat also are peroxidase positive [7].

Counting method. The grain counts of 100 monocytes per rat were recorded for each of the 16 time points studied. Non radioactive, simultaneously stained control preparations were included in all batches of autoradiograms and the grains over the nuclei of a total of 1000 leukocytes were counted. The background values thus ob-

tained for the different batches were used in correcting the proportion of labelled cells and the mean grain count per labelled cell by the method of Stillström [5]. The average background was 0.3 grains per leukocyte and did not exceed 0.5 grains per leukocyte in any of the 8 batches of autoradiograms.

Results

Controls of the normal steady state. The total blood leukocyte count in the rats was 8,500 ± 2,000 cells/mm³ of blood (mean ± SD of all values). Morphologically typical monocytes constituted 8.3 ± 2.1% of the leukocytes, or 700 ± 240 cells/mm³ (means ± SD of all values). When tested by a two-tailed Student's t test, neither the leukocyte count nor the absolute monocyte count showed any significant trend towards higher or lower values in any rat, and analysis of variance (one factor design) revealed no significant differences between the values for the different days of the observation period ($F = 1.88$ for leukocytes, $F = 0.70$ for monocytes). Myelocytes usually constituted 0.0–0.5% of the leukocytes, and the maximum observed was 1.4%.

Labelling of blood cells one hour after the first injection of ³HTdR. At one hour, the calculation method used [5] gave 12.2% labelled monocytes. However, in the second part of this investigation [6], 13% of the other cells in the same batch of autoradiograms were found to be falsely labelled. This shows that the background in the test preparations of this particular batch must have been greater than in the control preparations. Thus, the true labelling index of monocytes at one hour was 0%. This conclusion is also supported by the grain count distribution of the monocytes, which did not differ significantly from the average background distribution recorded in the whole experiment. Furthermore, none of the 600 monocytes scored showed more than 4 grains.

A few mononuclear blood cells with up to 30 grains were seen at one hour. As the 3,000 leukocytes analysed in the non-radioactive control preparations included one cell with 6 grains and one with 7 grains, only cells with 8 or more grains were considered obviously labelled. 17 such cells (0.06%) with a mean grain count of 14.5 were found when 28,410 leukocytes were examined in preparations stained by the combined method for peroxidase and lysozyme. All these labelled cells were large mononuclears varying in morphological appearance; about half were distinctly basophilic. In spite of the monocyte-like appearance of some of the cells, none of them showed any evidence of lysozyme or peroxidase activity.

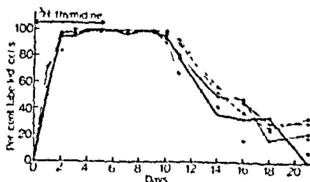


Fig 1 Percentage of monocytes labelled in the blood of 6 rats during and after intermittent injections of ³H thymidine

Percentage of labelled monocytes The percentage of monocytes labelled in the individual rats at each time point is shown in figure 1 On the first day, 58 3±4 0% (mean ± SE) of the monocytes were labelled, and 100% labelling was reached by day 4 In spite of the cessation of ³HTdR injections between days 5 and 6, 100% labelling persisted until day 9

The mean percentage of unlabelled monocytes decreased exponentially during the first 5 days of the experiment, as is shown by the closely linear decline in a semilogarithmic plot (fig 2) The half-time given by the estimating equation was 12 3 h, if this is multiplied by 1/ln 2, a turnover time of 17 7 h is obtained for the monocytes The mean percentage of heavily labelled monocytes, defined as cells with 16 or more grains, also showed an exponential decline after day 7 (fig 2) The calculations resulted in a half time of 13 6 h and a turnover time of 19 6 h

The influence of different background correction methods on the results for labelling percentage was great When labelled cells were defined as cells with 2 or more grains or as cells with 3 or more grains, significantly lower values were obtained than by the method of STILLSTRÖM [5] which allows the recognition of faintly labelled cells Calculation of the half-time of the disappearance of unlabelled monocytes resulted in 15 3 and 16 8 h, respectively, when the limits of 2 and 3 grains were used When the percentage of labelled monocytes in each rat on day 1 was estimated by the background subtraction method of ENGLAND *et al* [2] exactly the same values were obtained as by the method of STILLSTRÖM [5]

Grain count of monocytes The mean grain count per labelled monocyte in the individual rats at each time point is shown in figure 3 The

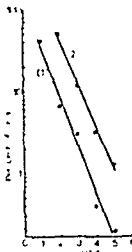


Fig. 2. Disappearance from the blood of unlabelled monocytes (●) during labelling and of heavily labelled (16 or more grains) monocytes (○) after labelling with intermittent injections of ^{3}H-thymidine. Regression line (1): estimating equation $\log Y = 2.12 - 0.3456\,X$, standard error of estimate 0.16. Regression line (2): estimating equation $\log Y = 2.17 - 0.3305\,X$, standard error of estimate 0.05.

mean grain count rose in each rat from the initial value on day 1 to a maximum that was approximately 4 times this value. The mean value for all rats rose from 3.9 (day 1) to 15.8 (day 5). The level reached on day 5 was maintained for 2 more days in spite of the cessation of the ^{3}HTdR injections (the mean was 14.8 on day 6 and 15.7 on day 7). Between days 7 and 11 the mean values showed a closely exponential decline (estimating equation $\log Y = 1.37 - 0.1878\,X$, standard error of estimate 0.03) with a half time of 1.6 days (Fig. 6b).

On day 4 a dip or levelling off of the mean grain count curve was seen. Its presence in every one of the 6 rats suggests that it was not caused by statistical factors only. A significantly greater number of monocytes with 1–12 grains was found on day 4 than on day 3.

The grain count distributions of labelled monocytes on days 5, 6 and 7 were very similar. When they were standardized with regard to the mean grain count, no significant difference was found between days 7 and 6 ($p > 0.01$) and only a slight difference existed between days 6 and 5 ($0.01 > p > 0.001$). When the grain count distributions on days 7–11 were

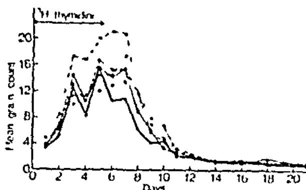


Fig 3 Mean grain count per labelled monocyte in the blood of 6 rats during and after intermittent injections of ³H thymidine

standardized with regard to the mean grain count and to the number of cells, a slight, though statistically significant, broadening of the distribution was seen

Discussion

The absence of labelled monocytes one hour after the first injection of ³HTdR shows that circulating rat monocytes do not synthetize DNA The 0 06% of leukocytes that were obviously labelled at this time point could be cytochemically differentiated from monocytes The presence of a similar number of labelled cells in normal human blood after *in vitro* labelling with ³HTdR was reported many years ago by Bond *et al* [1] and most of the cells found in the present study are probably identical with the group 2 and group 3 cells of these authors

The disappearance of unlabelled monocytes from the blood was exponential, indicating that monocytes leave the circulation at random Because the grain count data suggest that there is a storage pool from which old unlabelled monocytes can be released into the circulation after the time point when labelling is started the obtained turnover time of blood monocytes (18 h) is a maximum value

The fact that the disappearance of heavily labelled monocytes followed an exponential function very similar to that of the unlabelled cells speaks against the occurrence in this experiment of any significant reutilization of label and suggests that the labelling did not disturb the kinetics of the monocyte system

A model of the monocyte system involving only one precursor cell (i.e. the stem cell) would not account for a rise of the mean grain count to more than twice the initial value. The increase to 4 times the initial value that was observed can only be explained by a model in which 2 or more succeeding dividing precursors are assumed, the last (most mature) of them incorporating less label per phase of DNA synthesis than the earlier one(s). The half time of the mean grain count of labelled blood monocytes (1.6 days) is an upper limit of the average generation time of the precursors.

After the cessation of ³HTdR injections the mean grain count should immediately begin to fall as a result of the division of previously labelled precursor cells. It was, however, found that for 2 days the mean grain count and even the grain count distribution of labelled monocytes remained similar to those of day 5. The simplest interpretation is that monocytes formed by day 5 had been stored at their place of origin before being released into the blood on days 6 and 7. The concept of a storage pool where all mature or maturing monocytes stay before release into the circulation seems, however, to conflict with the rapid appearance of labelled monocytes in the blood at the beginning of labelling. Apparently, therefore, a proportion of the monocytes is released into the blood soon after the division of the immediate precursor, while another proportion is stored at the place of origin for a few days. A similar non-uniform behaviour of mouse monocytes has been suggested by VAN FURTH and DIESSELHOFF-DEN DULK [3]. If the storage pool follows the pipeline principle, the sudden appearance in the blood of monocytes with a low grain count on day 4 could probably be explained as the first release of labelled cells from the storage pool.

Acknowledgement. I wish to thank Dr. T. [illegible] for helpful [illegible] [illegible] this work. The investigation was supported by a grant from the [illegible] [illegible] Helvetic[illegible]

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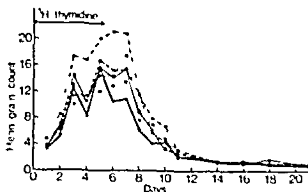


Fig 3 Mean grain count per labelled monocyte in the blood of 6 rats during and after intermittent injections of ³H thymidine

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Einfluss der neonatalen Thymektomie auf die virale Leukämogenese der Maus

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Abstract. Thymectomy of neonatal mice, which had been treated with Graffi virus, caused an inhibition of the leukemogenesis. The effect was dependent on the hematological character of the primary leukoses used as virus source. The greater the lymphatic proportion in the primary leukosis, the stronger was the inhibitory effect on leukemogenesis. The smallest inhibition of leukemogenesis was observed in thymectomized mice following injection of virus from chloroleukemias.

Key Words: Graffi virus · Leukemia viruses · Mouse leukemia · Thymectomy

Der Einfluss des Thymus auf die Leukämogenese virusbedingter Leukämien ist für bestimmte Modelle muriner Leukämien nachgewiesen worden [9, 11–13, 15–17]. Sollte sich die Ätiologie menschlicher Leukosen ebenfalls als virusbedingt erweisen, könnte auch in diesem Falle dem Thymus eine besondere Rolle im Verlauf der Leukämogenese zukommen.

In früheren Versuchen konnten bei bestimmten virusbedingten Leukämien die ersten leukämischen Veränderungen im Thymus beobachtet werden [1, 14]. Thymektomieversuche an adulten Tieren (Mäusen, Ratten, Hamstern) führten zu widersprüchlichen Ergebnissen im Hinblick auf die Leukämiequote. Übereinstimmende Aussagen über die Bedeutung des Thymus bei der Leukämogenese ergaben sich erst, als Gross [9], Miller [12, 13], Levinthal *et al.* [11], Chieco and Taylor [2] mit neugeborenen thymektomierten Tieren arbeiteten. Neonatale Thymektomie erwies sich als geeignetes Mittel, um der Frage nach der Targetzelle nachzugehen. Entsprechende Versuche am Virusmodell der myeloischen Leukämie der Maus [7, 8] standen noch aus.

Die von Graffi *et al.* [7, 8] beschriebene Leukämie der Maus nimmt infolge ihres myeloischen Charakters unter den bekannten viral bedingten

Mauseleukamien eine Sonderstellung ein. FEY und GRAFFI [4] und FEY [5] konnten die Bedeutung der Milz für die Genese der myeloischen Leukämie nachweisen. Splenektomie senkte die Anzahl der auftretenden Leukosen auf ein Funftel des Kontrollwertes; damit wurde die Bedeutung der Milz als Targetorgan nachgewiesen.

Die myeloische Leukämie zeigte im Verlauf der zellfreien Transplantationen eine Tendenz zur Aufsplitterung in hämatologisch verschiedenen Typen [6]. Dabei traten unter anderem – zwar selten – auch lymphatische Leukamien auf. Reifzellige myeloische sowie die seltenen lymphatischen Leukamien hatten nach zellfreier Transplantation die geringste Aufsplitterungstendenz, wahrend Virusmaterial aus unreifzelligen Leukamien die starkste hamatologische Aufsplitterung ergab.

Im vorliegenden ersten Teil der durchgefuhrten Thymektomieversuche wurde der Einfluss des Thymus auf die virale Leukämogenese derartiger hamatologisch differenter Leukamien untersucht.

Material und Methodik

Tiermaterial. Es wurden Mause des Inzuchtstammes XVII Berlin verwendet. Die Spontanleukämiequote liegt bei diesem Stamm um 1% [10]. Es wurde mit 1–3 Tage alten Neugeborenen gearbeitet. Die Tiere wurden mit Einheitspellets ernährt.

Thymektomie. Neugeborene Tiere wurden im Alter von 1–3 Tagen nach einer modifizierten Methode von DISHLER und RUDALI [3] thymektomiert. Als rationellste Anästhesie erwies sich die Äthernarkose. Die narkotisierten Tiere wurden auf einer vorgewärmten Petrischale befestigt, wobei darauf geachtet wurde, dass eine ungehinderte Flankenatmung möglich war. In der Hohe des Sternums erfolgte ein etwa 3–4 mm langer subkutaner Schnitt. Danach musste das Sternum bis etwa in die Höhe der dritten Rippe gespalten werden. Mit stumpf geschmolzenen Glaskanülen konnten die Thymusläppchen freigelegt werden. Die Thymuslappchen wurden mit gebogenen Glaskanülen vorsichtig nacheinander abgesaugt. Der erforderliche Unterdruck wurde mittels einer Wasserstrahlpumpe regulierbar erzeugt. Die Operation beobachteten wir unter dem Stereomikroskop bei 20facher Vergrosserung. Meistens lösten sich die Thymuslappchen in einem Stuck ab. Tiere, bei denen die Thymusläppchen wahrend des Absaugens einrissen, wurden gekennzeichnet. Nach Kontrolle auf Vollständigkeit der Operation wurden die Wundränder mit Akutol-Wundspray (Hersteller Spofa, Prag) bestrichen und zugeklebt. Diese Verschlussmethode hat sich gegenüber anderen Methoden gut bewährt. Nach dem Verschliessen der Wunde wurden die Tiere mindestens eine Stunde auf einer Wärmeplatte gewarmt. Bevor sie der Mutter zuruckgegeben wurden, erhielt diese etwas Wundspray an den Körper, um sie an den Fremdgeruch zu gewohnen.

Die Operationssterblichkeit verringerte sich mit zunehmendem Alter der Tiere. Sie lag durchschnittlich zwischen 5 und 10%.

Mangel an Kannibalismus wurde beobachtet. Nach dem Tode der Versuchstiere wurden Autopsien und mikroskopische Untersuchungen durchgeführt, um Thymusreste auszuschließen und damit die Vollständigkeit der Thymektomie zu sichern. Tiere mit Thymusresten wurden ausgesondert.

Gewinnung des Ausgangsmaterials und Leukämieübertragung. Nach der Auswahl der Ausgangsleukämien wurden G-4-Filtrate hergestellt und Neugeborenen injiziert. Die erneut auftretenden Leukosen des gesuchten hämatologischen Types wurden weiter transplantiert. Die Auswahl erfolgte über mehrere Generationen.

Nach der hämatologischen Differenzierung der Leukämien wurden leukämisch umgewandelte Thymi, Milz und Knochenmark dem Ausgangstier entnommen und mit PBS (Phosphatpuffer) im Homogenisator zerkleinert. Nach Abzentrifugation der Zelltrümmer (3000 U, 10 min) wurde der Überstand zweimal durch Schottsche G-4-Filter gesaugt. Die Injektion erfolgte unmittelbar nach der Filtratherstellung.

Hämatologie und Histologie. Die Tiere wurden ständig durch Palpation und hämatologische Kontrolle überwacht, dabei wurde aus den Schwanzgefäßen Blut entnommen. Die Blutausstriche wurden luftgetrocknet, 25 min in Methanol fixiert und anschließend mit Giemsa gefärbt. Gewebe- bzw. Organstückchen wurden in Formalin fixiert, in Paraffin eingebettet, geschnitten und mit Hämatoxylin-Eosin gefärbt.

Ergebnisse

Voruntersuchungen. Unbedingte Voraussetzung für vergleichende Thymektomieversuche war das Vorhandensein eindeutig differenzierter Ausgangsleukämien. Die hämatologische Differenzierung der verwendeten Leukämien erfolgte nach der von Fey und Graffi [4, 6] vorgenommenen Klassifizierung. Myeloische Leukämien, deren eindeutigste Form die Chloroleukämien sind, standen zur Verfügung. Durch hämatologische Kontrollen konnten einige lymphatische Leukämien gefunden werden. Durch weitere Transplantation und auf Grund ihrer relativ geringen Aufsplitterung in andere Formen konnten von 145 auswertbaren Tieren zu etwa 10% reine lymphatische Leukämien mit kurzer Latenzzeit (1–3 Monate) gewonnen werden. Diese lymphatischen Leukämien sind makroskopisch durch eine starke Vergrößerung des Thymus sowie der Lymphknoten (einschließlich der mediastinalen Lymphknoten) gekennzeichnet. Milz und Leber sind dagegen kaum oder nur mäßig vergrößert. Hämatologisch [illegible] Bild [illegible], wobei [illegible] im Reifungsgrad dieser Leukämien keine Differenzen auftreten. Neben Lymphoblasten [illegible] Lymphozyten fallen im Blutausstrich die für die lymphatischen Leukämien [illegible] auf.

Lymphatische [illegible] Myeloformen sowie Erythroblastenleukämien [illegible]

Tabelle 1 Befundquoten nach Injektion von Viren verschiedener Leukämien

	Auswertbare Tiere	Leukämien	
		Anzahl	%
Lymphatische Leukämien			
Scheinthymektomie	35	34	97
Kontrollen	45	45	100
Thymektomie	148	41	29
Kontrollen	231	218	94
Chloroleukämien			
Thymektomie	115	69	60
Kontrollen	112	95	85
Lymphatisch retikuläre Leukämien			
Thymektomie	62	24	37
Kontrollen	81	71	88
Erythroblastenleukämien			
Thymektomie	46	27	59
Kontrollen	47	41	87

gleichenden Thymektomieversuche zur Verfügung. Während die lymphatisch-retikulären Leukämien als echte Mischformen in unseren Selektionsversuchen öfter zu finden waren, kamen die Erythroblastenleukämien seltener vor.

Im weiteren Verlauf der Voruntersuchungen überprüften wir ein eventuelles Auftreten des Wasting-Syndroms. Das Wasting-Syndrom, ein Krankheitsbild, das sich bei neonatal thymektomierten Tieren zeigen kann und dessen Symptome unter anderem Wachstumshemmung und Gewichtsverlust sind, fand sich bei den nur operierten Tieren nicht. Von 35 auswertbaren, nur thymektomierten Tieren erreichten 28 ein Alter von über 10 Monaten. Die Tiere blieben im Wachstum zunächst zurück, wiesen aber nach 2–3 Wochen im Allgemeinzustand keinen Unterschied zu den Kontrolltieren auf.

Bei den späteren Versuchen erreichten viele Tiere nicht den körperlichen Bestzustand der Kontrolltiere. Es starben Würfe mit Symptomen der Kachexie. Der überwiegende Teil der Tiere aber zeigte kein strenges Wasting-Syndrom.

des Virus in thymektomierte Tiere eine unterschiedliche Entwicklung Bei 95% der Kontrollen erschienen die typischen Organvergrösserungen aber auch die thymektomierten Tiere wiesen vermehrt Befunde (60%) auf (Tab I) In den Kontrollversuchen war die durchschnittliche Latenzzeit für Befundtiere 129 Tage, wobei eine starke Streuung zu beobachten war Die durchschnittliche Überlebenszeit fur befundfreie, virusinfizierte, thymektomierte Tiere lag bei 172 Tagen Die durchschnittliche Latenz für thymektomierte, virusinfizierte Tiere mit Befund lag bei 161 Tagen

Wirkung der Thymektomie auf die Leukamogenese nach der Injektion von Virus aus lymphatisch retikularen Mäuseleukämien Kontrolltiere entwickelten Leukosen nach einer Latenzzeit von 144 Tagen Die Latenzzeiten der thymektomierten Tiere lagen fur befundfreie und Befundtiere zwischen 135 und 160 Tagen (Tab I)

Wirkung der Thymektomie auf die Leukamogenese nach der Injektion von Virus aus Erythroblastenleukamien Die mittlere Latenzzeit der Kontrollen lag bei 125 Tagen Thymektomierte, befundfreie Tiere hatten Latenzen von durchschnittlich 142 und thymektomierte Befundtiere von 144 Tagen (Tab I)

Hamatologische und histologische Befunde Als eine der ersten Folgen der Thymektomie zeigte sich eine Verarmung des Blutes an kleinen Lymphozyten Diese Lymphopenie blieb bei thymektomierten Tieren die ohne makroskopischen Befund starben, in einem bestimmten Masse erhalten Bei Kontrolltieren, die Virus aus selektionierten lymphatischen Leukamien erhielten, uberwogen bei den auftretenden Befunden lymphatische und lymphatisch-retikulare Formen Bei thymektomierten Tieren die Befunde zeigten, waren myeloisch-retikulare Formen vorherrschend Bei Kontrolltieren, die Virus aus Chloroleukamien injiziert bekommen hatten wiesen die Blutausstriche ein breiteres Spektrum von Leukamien auf es uberwog aber der myeloische Charakter der Leukamien

Bei den Befunden der Kontrolltiere, die G-4-Filtrat von lymphatisch retikulärem Ausgangsmaterial erhalten hatten zeigte sich ein Vorherrschen der lymphatisch-retikularen Formen Thymektomierte Befundtiere wiesen myeloisch retikulare und myeloische Leukamietypen auf

Nach der Injektion von Erythroblastenleukamien zeigten die Kontrolltiere uberwiegend myeloische Leukamietypen, die aber meist mit einer mehr oder weniger starken Erythroblastenkomponente vergesellschaftet waren Reine Erythroblastenleukamien traten nur in geringerem Masse auf Bei den Befundtieren unter den thymektomierten Tieren ergaben sich entsprechende Verhältnisse

Diskussion

Die Bedeutung des Thymus bzw. der Thymuszelle für den Ablauf der viralen Leukämogenese wurde durch die Ergebnisse der Thymektomie aufgezeigt. Nach der Übertragung von Virusmaterial aus selektionierten lymphatischen Leukämien in thymektomierte neugeborene Mäuse erhielten wir eine eindeutige Hemmung der Leukämogenese. Beim Vergleich der erhaltenen Befundquote mit entsprechenden Angaben anderer Autoren [9, 12, 13] kann man feststellen, dass unsere Werte in annähernd gleichem Bereich liegen. So beobachtete GROSS [9] z. B. nach Inokulation des Passage-A Virus in thymektomierte Neugeborene (C3H) bei 2% der Tiere Leukämien, während die Kontrollen zu 87% Leukosen aufwiesen. MILLER [12] führte entsprechende Versuche durch. Dabei ergaben sich für die Kontrollen Befundquoten von 18–100%, dagegen entwickelten sich nur bei 1–5% der thymektomierten Tiere Leukosen. GROSS [9], MILLER [12, 13], LEVINTHAL *et al.* [11], NAKAKUKI *et al.* [15] arbeiteten mit Leukämien, deren hämatologischer Charakter von Anfang an als lymphatisch definiert war, während in unseren Versuchen, von myeloischen Leukämien ausgehend, diese lymphatischen Ausgangsformen erst gewonnen wurden.

Leukosen, welche sich bei den thymektomierten Tieren entwickelten, die Virus aus selektionierten lymphatischen Leukämien erhalten hatten, waren vor allem myeloisch, retikulär bzw. es zeigten sich Mischformen. Bei den Kontrollen dominierte die lymphatische Komponente.

Die Leukämogenese der myeloischen Leukämie – in unserem Fall der Chloroleukämie – wurde durch Thymektomie schwach gehemmt. Die aufgetretenen Leukämien waren überwiegend myeloisch, retikuläre und Mischformen wurden beobachtet.

Nach den Untersuchungen über die Aufsplitterungen [6] bewirken Viruspräparationen aus ursprünglichen myeloischen Leukämien, zu denen die Chloroleukämie zählt, ein breites Spektrum der entstehenden Leukämietypen. Es ist wahrscheinlich, dass nach Injektion von Virus aus Chloroleukämien in thymektomierte Tiere der Anteil der eventuell entstehenden lymphatischen und lymphatisch-retikulären Leukämien gehemmt wird, so dass die aufgetretene Hemmung der Chloroleukämie nur schwach ist. Das differenzierte Auftreten der Hemmwirkung der Thymektomie bei der Leukämogenese von Mischleukämien spricht für diese Annahme.

Die Spezifität der Wirkung der Thymektomie wird durch die Ergebnisse der Schein-Thymektomie unterstrichen. Ein [illegible] des Organs

hatte keinen Einfluss auf dessen Rolle im Verlauf der viralen Leukämogenese.

Zusammenfassend lässt sich feststellen, dass die aus selektionierten lymphatischen Leukämien gewonnenen Viren sich hinsichtlich ihrer Beeinflussung durch Thymektomie nicht von den bekannten, lymphatische Leukämie erzeugenden Viren unterscheiden und dass auch in diesem System der Thymus die Targetzellen für die lymphatische Leukämogenese enthalten muss

Zusammenfassung

Thymektomie bei neugeborenen Mäusen, die Graffi-Virus injiziert bekamen verursachte eine Hemmung der viralen Leukämogenese Die Grösse der Hemmwirkung hing ab vom hämatologischen Charakter der Ausgangsleukosen Je grösser der lymphatische *Anteil in der Ausgangsleukose war, desto stärker war die Hemmung der* Leukämogenese Am wenigsten wurde die Leukämieentstehung in thymektomierten Mäusen nach Injektion von Virusmaterial aus Chloroleukämien gehemmt Scheinthymektomie hatte keinen Einfluss auf die Entwicklung der Leukämien

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Globin Chain Synthesis in Sickle Cell Trait Under Conditions of Folate Antagonism[1]

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Abstract Folic acid deficiency has been reported to produce a substantial change in the ratio of hemoglobin S to hemoglobin A in the blood of individuals having sickle cell trait Synthesis of the specific β-chains of these hemoglobins was studied before and after folate antagonism was produced by methotrexate administration. Incorporation of L leucine ^{14}C into β^A and β^S by bone marrow cells was unchanged as a result of the treatment with methotrexate indicating that synthesis of these globin chains was not selectively affected by the drug

Key Words
Folic acid
Hemoglobin S
Hemoglobin synthesis
Methotrexate
Sickle cell trait

In recent years, it has become apparent that nongenetic factors may in some cases significantly alter the ratio of normal and variant hemoglobin forms present in the blood of heterozygous individuals In each instance reported the effect appeared to be reversible

Iron deficiency has been shown to produce a significant decrease in the percentage of the variant hemoglobin both in individuals heterozygous for hemoglobin S [1] and for hemoglobin E [2] When iron therapy was instituted in these patients, the fraction of the abnormal hemoglobin rapidly increased to within the usual range for these hemoglobinopathies

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Folic acid deficiency has also been found to be associated with significant changes in the percentage of sickle hemoglobin in the blood of heterozygous individuals [3, 4]. In a patient with folic acid deficiency described by HELLER *et al.* [3] hemoglobin S comprised only about 10% of the total hemoglobin, but increased to approximately 40% after treatment with folic acid.

Case Report

A 10-year-old girl was admitted to the University of Illinois Hospital in 1970 because of generalized lymphadenopathy. Hemoglobin 10.8 g/100 ml, white cell count 6,900/mm³; differential count: 20% segmented neutrophils, 2% metamyelocytes, 3% lymphocytes, and [illegible]% lymphoblasts. A sickle cell preparation was positive and starch gel electrophoresis of hemolysates of her red cells at pH 8.6 [5] indicated the presence of sickle cell trait. Stained smears of aspirated bone marrow: hypercellularity with lymphoblasts making up [illegible]% of the nucleated cellular elements. Megakaryocytes were not seen and normoblasts were present in greatly reduced numbers.

A diagnosis of acute lymphoblastic leukemia was made and initial medications were begun consisting of allopurinol [illegible] mg daily, prednisone 2 mg/kg daily and vincristine 0.05 mg/kg once weekly. After a 4-week period bone marrow examination indicated complete hematologic remission. Megaloblastic changes were not observed. Subsequent antileukemic therapy consisted of oral methotrexate 2.5 mg daily.

The percentage of hemoglobin S in peripheral blood samples was determined by a [illegible] electrophoresis procedure [6] and remained at 4[illegible]% of the hemoglobins A and S throughout the course of the study. Prior to the initiation of methotrexate therapy, her urinary excretion of formiminoglutamic acid (FIGLU) for an 8-hour period following oral administration of 10 g of L-histidine was 7.0 mg (the normal range of values with this procedure [7] is [illegible] mg). A sample of aspirated bone marrow was incubated in a medium containing [illegible] as described previously [8]. Globin was prepared from an unfractionated cell lysate and subjected to carboxymethyl cellulose column chromatography [9] and the incorporated radioactivity in the β chains of hemoglobins A and S was determined. Incorporation of radioactivity into the 2 β chain fractions resulted in very nearly equal specific activity values for β^A and β^S as has previously been reported in studies of globin synthesis by bone marrow cells [1[illegible]].

At the end of a [illegible]-week period of receiving methotrexate, the [illegible] peripheral blood counts remained normal but a bone marrow examination indicated early evidence of leukemic relapse. The red cell precursors exhibited megaloblastic features and comprised approximately [illegible]% of the nucleated cellular elements of the marrow. Urinary excretion of FIGLU at that time was [illegible] mg/8 h, indicating a significant degree of folate antagonism. A determination of the relative rates of synthesis of the β^A and β^S chains by bone marrow cells was unchanged from the study done prior to the administration of methotrexate.

Discussion

In this study, globin synthesis was determined using bone marrow cells which would be expected to be most significantly affected by the folate antagonism, and synthesis of the individual β-chains which are the specific determinants of hemoglobins A and S was studied apart from the contribution of the α-chains. The latter are presumed to derive from a single pool of α chains for both hemoglobin types, and synthesis of the α-chains appears to be independent of the synthesis of the non-α globin chains [8].

The antileukemic effect of methotrexate appears to be attributable primarily to the activity of the drug as an inhibitor of dihydrofolate reductase [11]. FIGLU, which is a catabolic product of histidine, normally undergoes further catabolism by a reaction requiring tetrahydrofolate [12]. FIGLU accumulates and is excreted into the urine in substantial quantities in folate-deficiency states [13], and increased FIGLU excretion has previously been demonstrated to occur following the administration of methotrexate to patients with leukemia [14].

Although a significant increase in FIGLU excretion was documented in our patient following methotrexate administration, it is nevertheless conceivable that other biochemical changes that result from a dietary lack of folic acid may not be reproduced by the effect of methotrexate. Thus, a folate-dependent biochemical reaction not requiring a reduced form of folic acid as a cofactor might be affected by dietary folate deficiency but not by methotrexate. Within these limits of the experimental model, however, there does not appear to be a demonstrable effect of folate deficiency on the synthesis of the β globin chains of hemoglobins A and S in sickle cell trait.

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Glucose-6-Phosphate Dehydrogenase Toulouse

A New Variant with Marked Instability and Severe Deficiency Discovered in a Family of Mediterranean Ancestry[1]

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Abstract A new G-6-PD variant has been discovered in a 6-year old boy who is a carrier of a severe enzyme deficiency, with a typical favism background The patient is of South Italian ancestry, and the enzymatic anomaly has been transmitted by the maternal grandfather The enzyme is characterized by an electrophoretic mobility slightly slower than that of the Mediterranean group variants (nearly 100%) with a normal affinity for G-6-P and NADP The activity in red blood cells is very low (3% of the normal) The mutant enzyme is very unstable and its optimum pH is slightly biphasic It shows an increased utilization of deamino-NADP and 2-deoxyglucose 6-phosphate Since this variant is different from those previously described it has been named G 6-PD Toulouse

Key Words
Erythrocyte enzymes
Favism
G-6-PD deficiency
G-6-PD Toulouse
Hemolytic anemia
Mediterranean G-6-PD
Thalassemia

Recent progress in biochemical genetics has clearly demonstrated the large differences of glucose-6 phosphate dehydrogenase (G-6-PD) mutants expressed in man This enzyme defect in erythrocytes is usually revealed by hemolysis after administration of various drugs such as primaquine, sulfamides and others, or absorption of fava beans [3] In several cases, especially in white people chronic hemolytic anemia can be observed even without the administration of drugs [3, 17] However

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more than 40 of the 100 G-6-PD variants [28] identified in human populations today are not accompanied by any hematological manifestation since the enzyme activity is normal or is moderately reduced in most cases. The discovery of a variant of this latter group is the result of systematic investigations undertaken in different ethnic groups. Although the clinical severity of hemolysis does not always correspond to the degree of enzyme deficiency, it is related to the physiological activity of the enzyme under the influence of metabolites and coenzymes in red blood cells [29]. Among the molecular forms of enzymes susceptible to acute hemolysis caused by drugs or beans, the Mediterranean type variant occupies a predominant place. This variant is, in fact, one of the first recognized G-6-PD mutations. It is found not only in a great majority of the populations of the Mediterranean areas, but also in the near of Middle East, and in Asia.

In this paper, the authors report the study of a new G-6-PD variant associated with favism found in a family of Calabrian ancestry (South of Italy) settled in France for 2 generations.

Materials and Methods

Laboratory procedures. Standard laboratory procedures were used for hematological determinations. Hemoglobin A_2 was assayed electrophoretically on starch gel at pH 8.6 and on cellogel at pH 9.2. Tests for osmotic fragility and resistance to alkaline denaturation were carried out in accordance with the techniques outlined by [illegible] et al. [8].

G-6-PD activity of the hemolysates was assayed in a system containing triethanolamine buffer, pH 7.6 [illegible] 10 [illegible] M ethylenediamine tetraacetic acid (EDTA) [illegible] glucose-6-phosphate (G-6-P) [illegible] M and NADP [illegible] 10 [illegible] M. The reaction was followed spectrophotometrically at 340 nm, and the results are expressed in micromoles of NADP reduced per minute per [illegible] of red cells [illegible]. The level of reduced glutathione was estimated according to the [illegible] and [illegible] [illegible] method [illegible].

After partial purification by chromatography on [illegible] G-6-PD was characterized by the method recommended by the WHO [illegible] Committee [illegible]. The following investigations were carried out after dialysis of the partially purified G-6-PD.

(1) The [illegible] of the [illegible] mobility on [illegible] of normal G-6-PD and of the variant on 3 buffer systems: phosphate pH 7.0 [illegible] Tris-HCl pH 8.8 [illegible] and Tris-EDTA-borate, pH 8.6 [illegible]. (2) The Michaelis constant (K_m) for G-6-P and NADP. (3) The [illegible] rate of substrate analogues [illegible] G-6-P [illegible] NADP expressed as the percentage of the rate of [illegible] the [illegible] G-6-P or NADP. (4) The [illegible] of pH [illegible]

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Abstract A new G-6-PD variant has been discovered in a 6-year-old boy, who is a carrier of a severe enzyme deficiency, with a typical favism background The patient is of South Italian ancestry, and the enzymatic anomaly has been transmitted by the maternal grandfather The enzyme is characterized by an electrophoretic mobility slightly slower than that of the Mediterranean group variants (nearly 100%) with a normal affinity for G 6-P and NADP The activity in red blood cells is very low (3% of the normal) The mutant enzyme is very unstable and its optimum pH is slightly biphasic It shows an increased utilization of deamino-NADP and 2-deoxyglucose-6-phosphate Since this variant is different from those previously described it has been named G-6-PD Toulouse

Key Words
Erythrocyte enzymes
Favism
G-6-PD deficiency
G-6-PD Toulouse
Hemolytic anemia
Mediterranean G-6-PD
Thalassemia

Recent progress in biochemical genetics has clearly demonstrated the large differences of *glucose 6-phosphate dehydrogenase (G 6 PD)* mutants expressed in man This enzyme defect in erythrocytes is usually revealed by hemolysis after administration of various drugs such as primaquine, sulfamides and others, or absorption of fava beans [3] In several cases, especially in white people chronic hemolytic anemia can be observed even without the administration of drugs [3 17] However

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more than 40 of the 100 G-6-PD variants [28] identified in human populations today are not accompanied by any hematological manifestation since the enzyme activity is normal or is moderately reduced in most cases. The discovery of a variant of this latter group is the result of systematic investigations undertaken in different ethnic groups. Although the clinical severity of hemolysis does not always correspond to the degree of enzyme deficiency, it is related to the physiological activity of the enzyme under the influence of metabolites and coenzymes in red blood cells [29]. Among the molecular forms of enzymes susceptible to acute hemolysis caused by drugs or beans, the Mediterranean type variant occupies a predominant place. This variant is, in fact, one of the first recognized G-6-PD mutations. It is found not only in a great majority of the populations of the Mediterranean areas, but also in the near of Middle East and in Asia.

In this paper, the authors report the study of a new G-6-PD variant associated with favism found in a family of Calabrian ancestry (South of Italy) settled in France for 2 generations.

Materials and Methods

Laboratory procedures. Standard laboratory procedures were used for hematological determinations. Hemoglobin A_2 was assayed electrophoretically on starch gel at pH 8.6 and on cellogel at pH 9.2. Tests for osmotic fragility and resistance to [illegible] were carried out in accordance with the techniques outlined by [illegible] et al. [illegible]

G-6-PD activity of the hemolysates was assayed in a system containing [illegible] buffer, pH 7.6 [illegible] M, [illegible] and [illegible] (EDTA) [illegible] M, glucose-6-phosphate (G-6-P) [illegible] M and NADP [illegible] M. The reaction was followed spectrophotometrically at 340 nm, and the results are expressed in [illegible] of NADP reduced per minute per [illegible] of red cells [illegible]. The level of reduced glutathione was measured according to the [illegible] and [illegible] method [illegible].

After partial purification by chromatography on [illegible] gel, the G-6-PD was characterized by the method recommended by the WHO [illegible] [illegible]. The following investigations were carried out after [illegible] of the partially purified G-6-PD:

(1) The comparative electrophoretic [illegible] on starch gel of normal G-6-PD and of the variant, in 3 buffer systems: phosphate pH [illegible], Tris-HCl pH [illegible] and Tris-EDTA-borate pH 8.6 [illegible]. (2) The Michaelis constants (K_m) for G-6-P and NADP. (3) The [illegible] rate of substrate analogues (2-deoxy-G-6-P, [illegible] NADP) expressed as the percentage of the rate at which the same [illegible] of enzyme [illegible] G-6-P or NADP. (4) The [illegible] of pH [illegible].

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Glucose-6-Phosphate Dehydrogenase Toulouse

A New Variant with Marked Instability and Severe Deficiency Discovered in a Family of Mediterranean Ancestry[1]

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Abstract. A new G-6-PD variant has been discovered in a 6-year-old boy, who is a carrier of a severe enzyme deficiency, with a typical favism background. The patient is of South Italian ancestry, and the enzymatic anomaly has been transmitted by the maternal grandfather. The enzyme is characterized by an electrophoretic mobility slightly slower than that of the Mediterranean group variants (nearly 100%) with a normal affinity for G-6-P and NADP. The activity in red blood cells is very low (3% of the normal). The mutant enzyme is very unstable and its optimum pH is slightly biphasic. It shows an increased utilization of deamino-NADP and 2-deoxyglucose-6-phosphate. Since this variant is different from those previously described, it has been named G-6-PD Toulouse.

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Materials and Methods

Laboratory procedures. Standard laboratory procedures were used for hematological determinations. Hemoglobin A_2 was assayed electrophoretically on starch gel at pH 8.6 and on cellogel at pH 9.2. Tests for osmotic fragility and resistance to alkaline denaturation were carried out in accordance with the techniques outlined by Cabannes *et al.* [8].

G-6-PD activity of the hemolysates was assayed in a system containing tris-hydrochloride buffer, pH 7.6 [illegible] ethylenediamine tetraacetic acid (EDTA) [illegible] glucose-6-phosphate (G-6-P) [illegible] and NADP [illegible]. The reaction was followed spectrophotometrically at 340 nm, and the results were expressed in micromoles of NADP reduced per minute per [illegible] of red cells [illegible]. The level of reduced glutathione was estimated according to the [illegible] method [illegible]

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Table I Hematological values found in the two carriers of G-6-PD variant (hemizygous)

	RBC × 10^6/μl	Hemoglobin g/100 ml	PCV %	MCV μm³	MCH pg	MCHC %	MCF g/l NaCl
Normal range	4.2–5.7	13–18	38–52	83–98	27–32	32–36	4.6–3.6
Propositus	4.79	13.30	39.48	80	27.84	34.8	4
Maternal grandfather	5.475	12.18	39.51	69.2	22.2	32.2	3.25

Propositus G-6-PD deficiency alone Maternal grandfather combined thalassemia and G-6-PD deficiency

RBC = Red blood cells PCV = packed cell volume, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin concentration, MCF = median corpuscular fragility

dent G-6-PD activity in the range 5.5–10.5 The enzyme activity at each pH value was expressed as a percentage of maximum activity measured (5) Heat stability after 20-min incubation at 47 °C in presence of NADP and bovine albumin at a final concentration of 1 mg/ml

The Propositus and His Family The propositus Alain L., was a 6-year-old boy when biochemical and hematological studies were done in Toulouse From his past record he was affected by an acute hemolytic accident after beans absorption at the age of 2 years At the time, the patient was treated in a regional hospital near Toulouse, but no correlation was established with a metabolic disorder The diagnosis of G-6-PD deficiency was ascertained some years later, when the sister of the propositus who was 11 months old presented a similar hemolytic episode The young girl and her brother were sent for a complete examination at the Institute of Hematology (Prof R Biermé) in June 1970

The propositus was then in good condition He showed normal blood count (table I) Hemoglobin electrophoresis and bilirubin were normal A marked deficiency of G-6-PD was found 0.018 μmol of NADP reduced per minute per milliliter of red blood cells (normal 1.20 ± 0.17) Reduced glutathione was slightly diminished 49 mg/100 ml of red cells (normal range 55–80 mg)

Other members of the family were subjected to the same examinations (fig 1) G-6-PD assays done on blood of several members of the family over 3 generations are given in figure 1 The maternal grandfather Ern P., 66 years old suffers from the same severe deficiency as his grandson (table I) The great grandparents were natives of Calabria in the South of Italy The grandfather migrated to France and he married a French girl His 3 daughters were married to Frenchmen Although all 3 of his daughters should be heterozygous of the variant G-6-PD the red cell enzyme activity of 2 of them was nearly normal and only the propositus mother indicated a significant deficiency This may be related to the coexistence of thalassemia in these subjects

In addition to the G-6-PD anomaly a thalassemia trait has been discovered in the grandfather in his 3 daughters and in 2 of his grandchildren but not in the pro-

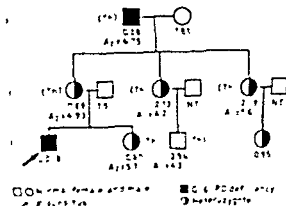


Fig. 1. Pedigree of the family. Subscript numbers represent G-6-PD activities expressed in µmoles of NADP reduced per minute and per m[illegible] of RBC, measured in each member. A_2 = Hemoglobin A_2 content (expressed in percentage) found in the carriers of thalassemia trait (Th). Note that the levels of G-6-PD activity observed in the propositus' aunts are higher than those expected in heterozygous individuals; this could be related to thalassemia trait. NT = Not tested.

[illegible] The hemoglobin A_2 values measured in the different subjects are mentioned in figure 1.

The other members of the family, one brother, 2 [illegible] of the grandfather, and the children were found to be quite normal.

Results

Table II gives the biochemical characteristics of the G-6-PD variant. Characterization yielded [illegible] results in the propositus and his maternal grandfather. The enzyme shows the following particularities: (1) A very marked instability. There was no detectable activity after 20-min incubation of the enzyme at 4[illegible] C, whereas normal G-6-PD has only a 10 to 20 percent reduction of activity. (2) The Michaelis constant for physiological substrates does not vary in relation to normal enzyme. (3) Altered substrate [illegible] of deamino-NADP and 2-deoxy glucose-6-phosphate [illegible]. (4) The pH optimum curve is slightly biphasic. (5) The [illegible] is slightly slower than that of normal G-6-PD [illegible] ranging between 96 and 100 [illegible] (fig. 2).

Table II. Characterization of glucose-6-phosphate dehydrogenase Toulouse

Enzyme type	Red cell enzyme activity, % of normal	Electrophoresis			K_m G-6-P μM	K_m NADP μM	pH curve	Utilization		Heat stability
		EDTA	Tris-HCl	phosphate				2d G 6-P	deamino-NADP	
Normal B	100	100	100	100	50–70	2.9–4.4	normal truncate	<4	55–60	normal
Toulouse	3	97	100	96	55	1.2–3	slightly biphasic	13	220	very low



Fig. 2. Comparative electrophoretic mobility of purified G-6-PD on starch gel. A = Phosphate buffer, pH 7.00 (25 V/cm, 18 h at + 4 °C): 1 and 5 G-6-PD B +, 2 and 4 G-6-PD Toulouse, 3 slow Negroid variant. B = TEB buffer (Tris, EDTA, borate), pH 8.6 (15 V/cm, 18 h at + 4 °C): 1, 3, 5 and 7 variant G-6-PD Toulouse, 4 and 8 G-6-PD B +, 2 and 6 slow Negroid variant. C = Tris-HCl buffer pH 8.8 (25 V/cm, 18 h at + 4 °C): 1 and 5 G-6-PD B +, 3, 4, 6 and 7 G-6-PD Toulouse, 2 and 8 slow Negroid variant.

Discussion

A comparison has been made between the present variant and the other variants previously identified. Considering the degree of deficiency of the 2 hemizygotes, it is clear that this variant must belong to the category of severe enzyme defect [28]. It is easy to discard variants with fast or very slow electrophoretic mobility. The G-6-PD which combines the normal electrophoretic mobility or slightly slower than that of the usual enzyme and a severe deficiency, are interesting for the discussion (table III). The values of Michaelis constants for G-6-P and NADP, and the substrate specificity make it possible to discard G-6-PD Mediterranean, Corinth, Orchomenos [28], Oklahoma [12], Milwaukee [28], Albuquerque [2], Ramat Gan and Ashdod [20]. G-6-PD Duarte [2] and Chicago [11] are clearly distinct by their optimum pH and the rate of utilization of 2-deoxy G-6-P. G-6-PD Alhambra [4] is isolated by its slow electrophoretic mobility at pH 7.0 and low rate of utilization of 2-deoxy G-6-P. Another variant found in France, G-6-PD Clichy [4], with a severe enzyme deficiency, though not fully characterized, stands out by its high K_m for G-6-P and its optimum pH in the alkaline range. Among the Asian variants, G-6-PD Hong Kong [24] and Indonesia [14] show a particularly lower K_m for G-6-P and a better heat stability. The optimum pH of

G-6-PD Hong Kong [26] is different from that of the present variant. G-6-PD Panay [8] is similar to the present variant in many characteristics but the rate of utilization of 2-deoxy G-6-P of Panay is significantly lower than that of the present variant. The rate of utilization of deamino-NADP differentiates G-6-PD Bangkok [24]. None of the Thai variants recently reported (G-6-PD Mahidol, Siriaj, Kan and Anat) are likely to be identical to the present variant [18].

Therefore, it appears, beyond reasonable doubt, that this variant enzyme is unique on the basis of its physicochemical parameters and the ethnic origin of the family. It is suggested that this variant should be designated G-6-PD Toulouse.

The study of the segregation of this mutation in the family tree is compatible with sex-linked heredity, as in all other variants described up to now (fig. 1). This family provides additional arguments to 2 series of genetic problems which often occur in populations of Mediterranean ancestry: (1) the combined hematological effects of G-6-PD deficiency and thalassemia; (2) the considerable heterogeneity of mutants in human groups suffering from favism.

Referring to the first problem, our results agree with those obtained by Fiorelli and Siniscalco [19] in their study of the inhabitants of a Sardinian village. They have shown that in carriers of the thalassemia trait and G-6-PD deficiency, the 2 genetic diseases had cumulative hematological effects. In these patients, at the level of peripheral blood, those 2 mutations tend to compensate each other. Thus, in the propositus' grandfather, we find the following hematological symptoms similar to those described by Fiorelli and Siniscalco [19]: (a) normal erythrocytic numeration; (b) slight decrease of hematocrit and of hemoglobin content, and (c) reduction of mean corpuscular volume and of mean corpuscular hemoglobin (table I). The latter reduction, however, is less marked than that observed in carriers of the thalassemia trait alone. These hematological signs are not, on the other hand, very evident in the propositus suffering from G-6-PD deficiency alone. It should be mentioned that G-6-PD deficiency is less severe in the thalassemia trait (grandfather) than in the propositus.

The pattern of association of G-6-PD Mediterranean with favism is not clear since some of the variant carriers have the favism problem while others have no problem. As suggested by Stamatoyannopoulos et al [22] besides the G-6-PD Mediterranean deficiency there may be an unknown genetic prerequisite in the hemolysis caused by fava

Table III Comparison of the biochemical characteristics of G 6-PD Toulouse with the more common variants and with those of similar electrophoretic behavior and enzyme activity

G 6-PD	Red cell enzyme activity, % of normal	Electrophoresis, % of normal			K_m G 6-P, µM	K_m NADP, µM	pH curve	Utilization		Heat stability
		EDTA	Tris-HCl	phosphate				2 d G 6-P	deamino-NADP	
Normal	100	100	100	100	50 70	2 9–4 4	normal	< 4	55 60	normal
Mediterranean	0–7	99–100	100	98–100	19 26	1 2–1 6	truncate biphasic	23–27	350	low
Orchomenos	0 7	100	100	92 94	11	2 1	biphasic	105	350	–
Corinth	0–7	100	100	100	19–26	1 2–1 6	biphasic	23 27	55 60	low
Oklahoma	4–10	–	100	–	127–200	20	peak at 8 2	~4	–	low
Milwaukee	0 5		92		224	–	peak at 8	3 7	–	–
Bat Yam	0	100	–	–	27	–	biphasic	40 45	–	very low
Ramat Gan	0	90 92			35	–	biphasic	40	–	very low
Ashdod	10	90 92			100		biphasic	40	–	slightly low
Albuquerque	1	100	100	100	115	11	sharp peak at 8 5	0	–	very low
Duarte	8 5	100	100	100	58	5	7 0	5 4	–	very low
Chicago	9 26		100		58–76	3 1–3 7	normal	~4		very low
Alhambra	9 20	96	95	85	55	2 6	–	2		low
Clichy	2	(acrylamide Tris pH 8 0)	100		178	–	plateau 9 10	–	–	slightly low
Indonesia	~5		100		25 52	4 9	slightly biphasic			slightly low
Hong Kong	0 15	100	–	–	1/2 normal	normal	normal	slightly increased		normal
Siriaji	5–15	100	–	100	18 27	–	biphasic	13 16	126–176	low
Panay	5	–	96	96	30	4 7	biphasic	~4		slightly low
Bangkok	5	100	100	100	60	5 3	8–8 5	8 4	40	very low
Toulouse	3	97	100	96	55	1 1–3	slightly biphasic	13	220	very low

G-6-PD Hong Kong [26] is different from that of the present variant. G-6-PD Panay [8] is similar to the present variant in many characteristics but the rate of utilization of 2-deoxy G-6-P of Panay is significantly lower than that of the present variant. The rate of utilization of deamino-NADP differentiates G-6-PD Bangkok [24]. None of the Thai variants recently reported (G-6-PD Mahidol, Siriraj, Kan and Anat) are likely to be identical to the present variant [18].

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Indonesia	*<5*		*100*		25–52	4–9	slightly biphasic			slightly low
Hong Kong	0–15	100	–	–	1/2 normal	normal	normal	slightly increased		normal
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beans Alternatively, 'G 6-PD Mediterranean' might not be a single genetic defect, and could be subdivided into several variants, some associated and others not associated with favism and drug sensitivity In fact, KIRKMAN *et al* [13] and STAMATOYANNOPOULOS *et al* [23] *have shown* that the variant originally classified as G-6 PD Mediterranean should be differentiated into several common variants (G-6-PD U-M, Orchomenos, Athens like and Corinth) which are distinguishable by electrophoresis and kinetic analysis The relationships between these variants and favism have not yet been examined G 6-PD Toulouse reported in this paper is associated with favism, and similar to G 6 PD Mediterranean in many characteristics, *although the 2 variants are definitely not identical It is* conceivable that this variant is common among Mediterranean subjects associated with favism

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Acta Haemat 51 250–255 (1974)

First Report of Hb E in Italy

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Abstract A case of heterozygosis for Hb E (*β26 [B8] glu→lys*) in a 39 year old woman with iron deficiency anaemia and hypothyroidism is described. Iron therapy raised the Hb E content from 17 to 33%. Hb E is frequent in Southeast Asia and occasionally reported in Europe. This case is the first observed in Italy.

Key Words
Haemoglobin E
Haemoglobinopathies
Iron deficiency and Hb E

Hb E was first noted in 1954 [1, 2] and its molecular structure was identified in 1961 [3]. Its geographical distribution, electrophoretic mobility, diminished oxygen affinity, and biochemical and clinical features have been fully studied [4, 5]. Wasi *et al* [6] reported that it made up 25–30% of the total haemoglobin in their large series. Concomitant α- or β-thalassaemia is often observed. Whereas association with the β-form leads to a percentage increase in abnormal haemoglobin, values decline between 17 and 25% in the presence of α-thalassaemia [6]. A decrease of this kind may be due to iron deficiency and its administration is often accompanied by a return to higher Hb values [7]. It has been observed that Hb A_2 also decreases in subjects with sideropenic anaemia, and iron deficiency may often mask increased Hb A_2 values in heterozygote β thalassaemia [7]. This paper describes the first Italian case of Hb E.

Material and Methods

The patient, a 39 year old woman from Rovigo with hypothyroidism and sideropenic anaemia, her widowed mother and 4 siblings were examined.

Standard blood tests were run, and osmotic resistance was determined with Simmel's method. Cellulose acetate electrophoresis was carried out with a pH 8.6 glycine buffer. After elution with distilled water, the fractions were read spectrophotometrically at 415 nm [9].

The abnormal haemoglobin was separated by chromatography on a 2.5×100 cm Sephadex DEAE A-50 column, using the technique of Huisman and Dozy [10]. Following urea concentration, the α and β chains were separated by incubation with p-chloromercuribenzoate (PCMB) [11] and examined electrophoretically on cellulose acetate. Fingerprints were taken on total globin and on the aminoethylated (AE) α and β chains [11, 13]. Abnormal peptides were eluted with 6 N HCl and hydrolysed at 110°C for 18 h, followed by examination in an Optica amino acid analyser.

Sickling was determined with 2% sodium metabisulphite. Solubility, alkali resistance, heat lability, and formation of methaemoglobin were evaluated by the methods of Itano [14], Betke et al. [15], Dacie et al. [16], and Evelyn and Malloy [17], respectively. Absorption spectra between 450 and 650 nm were carried out with a pH 6.8 1/15 M phosphate buffer, as described by Desmaret [18]. Prolonged exposure of the haemolysate to atmospheric air was used to obtain oxygenation, while deoxyhaemoglobin, methaemoglobin and cyanmethaemoglobin were obtained by adding sodium metabisulphite, ferricyanide, cyanide and potassium ferricyanide.

Results

The patient's widowed mother and 4 siblings were all completely normal. The haematological data of the patient are given in table I. The patient also suffered from hypothyroidism and iron deficiency anaemia. Plasma bilirubin 1.0 mg% conjugated, 0.5 mg% unconjugated. Simmel test (0.35% NaCl)+. There was no enlargement of liver or spleen. The Hb I rose from 17 to 33% after iron therapy (fig. 1, 2). PCMB dissociation revealed both slow moving β and normal α chains.

The globin fingerprint (fig. 3) was characterized by replacement of the usual positive arginine βT III by two new peptides. Of these, the anodic peptide was partly superimposed on the normal positive arginine αIV, while the cathodic peptide lay between α and βXIV. The fingerprints of the isolated and aminoethylated α and β chains showed that the former were normal, whereas βT III was replaced by two new peptides as well apart from the other tryptic fragments. The cathodic peptide reacted positively to arginine-specific staining while the anodic fragment did not react to the usual stains for tyrosine, tryptophan, histidine, arginine and methionine. Table II shows the analysis of these two new fragments. By comparison with normal βT III, they have one glutamic acid instead of two and a leucine residue.

Table I

	Hb Electrophoresis %			Hb g %	RBC, ×10⁶/µl	PCV, %
	E	F	A			
Before iron therapy	17	–	83	9.0	3.4	29
After iron therapy	33	–	67	12.2	4.2	38

[1] A = anisocytosis, P = poikilocytosis, T = target cells, M = microcytosis

The normal βIII sequence contains the following amino acids

VAL – ASN – VAL – ASP – GLU – VAL – GLY – GLY – GLU – ALA – LEU –
18 19 20 21 22 23 24 25 26 27 28
GLY – ARG
29 30



1 *2*

Fig. 1 Cellulose acetate electrophoresis (pH 8.6). From left to right: a normal subject, the patient before iron therapy, two normal subjects.

Fig. 2 Cellulose acetate electrophoresis (pH 8.6). From left to right: β thalassaemia trait, normal subject, the patient after iron therapy, heterozygous for Hb D

Table I (continued)

MCV, μm^3	MCHC, %	Retic., %	Red cell morphology: A	P	T	M	Plasma iron, μg%
88	31	1	+	±	±	+	45.0
92	32	0.7	±	–	–	–	93.8

Table II. Amino acid composition of the new peptides compared with normal βIII

	Amino acid residues		
	normal βIII	anodal abnormal fragment	cathodal abnormal fragment
Aspartic acid	2	1.1	
Glutamic acid	2	1.1	-
Glycine	3	2.0	1.1
Alanine	1	-	1.0
Valine	3	2.8	
Leucine	1	-	1.0
Lysine		1.1	-
Arginine	1		1.0



Fig. 3. Fingerprint (pH 6.4) of the abnormal chains. 1 = Absence of normal βIII; 2 = new anodic peptide; 3 = new cathodic peptide.

Lysine replacement may occur at position 22 or 26. The first is that of Hb E Saskatoon [19], the second that of Hb E. In our case, the cathodic peptide manifested only the four amino acids (alanine, leucine, glycine, and arginine) found as residues in position 27–30. Replacement thus involved position 26, and the mutation was typical of Hb E: β26 glu→lys.

Sickling was negative, alkali resistance, ferrohaemoglobin, heat lability, methaemoglobin formation, and the oxy-, deoxy- and cyanmethaemoglobin absorption spectra were all normal.

Discussion

Haemoglobin E is frequent in Southeast Asia and has been occasionally observed in Europe, though never in Italy. In our case it was associated with iron deficiency anaemia and hypothyroidism. Its initially low percentage value may be attributed to double heterozygosis between it and α-thalassaemia or the presence of iron deficiency. The fact that iron therapy led to a virtually 100-percent increase in Hb E suggests that its synthesis had been depressed by iron deficiency. Hypothyroidism was still present when this increase was obtained and would appear to be of no pathogenetic importance. In the absence of data for the father, it cannot be assumed that the patient was the original source of the mutation, especially since the possibility of remote Asiatic descent could not be excluded.

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Varia

Haemoglobin D Punjab

Remarks on the article of A. URIARTE, R. P. ATENCIO and B. COLOMBO, Acta haemat. *50*: 315–320 (1973). In Hb D Punjab, glutamic acid is replaced by glutamine at position 121 of the β chain [1]. The authors mentioned in their article that 'aspartic acid is substituted for an asparagine in position 121 of the β chain'. I would like to add on this occasion that we have observed a child with Hb S D disease who also had severe haemolytic anaemia [2] and its Hb D was identified as Hb D Punjab [3].

SINASI ÖZSOYLU, MD, Professor of Pediatrics
Hacettepe University, *Ankara* (Turkey)

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Istanbul Meetings 1974

(1) 9th Congress of the World Federation of Haemophilia
20th–22nd August, 1974
(2) Symposium on Platelet Preservation and Transfusion
23rd August, 1974. Organized by ISBT
(3) International Symposium on Blood Platelets
24th–27th August, 1974. Organized by O. N. ULUTIN
(4) International Symposium on Abnormal Haemoglobins and Thalassaemia
24th–27th August, 1974. Organized by M. AKSOY

All *meetings* will be held under the auspices of the Turkish Society of Haematology.

Correspondence should be addressed to Congress Bureau, VIP Tourism Ltd., Cumhuriyet Cad. 12, Elmadag, *Istanbul 4* (Turkey).

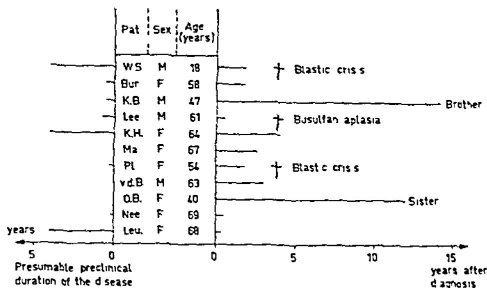


Fig. 1. Patients and follow up

reported. Haemostatic studies included bleeding time, recalcification time, one stage prothrombin time and fibrinogen levels. Treatment consisted of cytostatic agents such as Thio-Tepa and busulfan, ^{32}P, oral anticoagulants and iron.

Results

In figure 1, the 11 patients are presented with the assumed preclinical duration of the disease and the follow-up period after diagnosis has been made. During this time span, no other disease that could explain the thrombocytosis was found. The symptoms most regularly present were fatigue and a painful burning sensation in the finger tips and toes. One patient had intermittent claudication of the legs. Eight patients had bleeding episodes (73%). They were predominantly in the gastrointestinal tract, but there were also severe nose-bleeding, cutaneous bleedings and one patient had a cerebral haemorrhage. Four patients had thromboembolic complications (36%). Splenic enlargement by palpation, X-ray or scanning was found in 10 patients (90%), hepatomegaly in 6 (55%).

All patients had severe thrombocytosis, mean 1,400,000 mm³, range 800,000–3,200,000/mm³ (normal 150,000–350,000/mm³). The predominant morphologic aberration were giant platelets. 9 patients (82%)

had a neutrophil leucocytosis, mean 17,000/mm³, range 11,000–36,000/mm³. Frequently, eosinophilia and basophilia were seen. It reached 10 and 24% respectively. Occasional immature myeloid cells were found in 7 instances (63%). 9 patients (82%) were anaemic. The anaemias were hypochromic and probably due to chronic blood loss.

Initial data of haemostatic investigations were normal in all cases except two slightly prolonged bleeding times and one pathological prothrombin time (patient W. S.).

The leucocyte alkaline phosphatase at the time of diagnosis was variable. It was markedly above 150 in 5 (45%), moderately elevated (100–150) in 1, normal in 2 and decreased in 3 (normal values 20–80).

Bone marrow aspirations were studied at the time of diagnosis in 10 instances. All of them had a marked increase of megakaryocytes. Hypersegmentation of the megakaryocytes was a frequent finding and there was a very active erythro- and myelopoiesis in all instances. Two patients had a megaloblastic erythropoiesis with many sideroblasts. Initial trephine biopsies were performed in 9 patients. In all instances the megakaryopoiesis was increased and 4 patients had an increase of reticulin fibres.

Cytogenetic examination of the bone marrow cells was performed in 8 instances. In 7 cases it was normal. One patient had a Philadelphia chromosome. Thio-Tepa was effective in controlling thrombocytosis, at least temporarily, in most instances. The dose was 15 mg i.v. once or twice weekly up to a total dose of 120 mg. Intermittent busulfan was given orally in a dose of 2–4 mg/day to refractory cases. The longest remissions with Thio-Tepa were 10 months (patients K. B. and K. H.), the longest one with busulfan 34 months (patient K. B.). One patient (Lee) died of busulfan aplasia.

Cases of Particular Interest

Patient K. P. (a caucasian) was first seen in 1977 with [illegible] pain [illegible] in the fingers and toes [illegible] legs. His past history was [illegible] for a [illegible] fracture of the [illegible] in 1960. We found a slight hepatosplenomegaly, a hypochromic iron-deficiency anaemia (Hb 8 g%), 13,000 leucocytes/mm³ with a slight basophilia and 2.36 [illegible] platelets/mm³.

[illegible] determinations were [illegible] in our patients the [illegible] could not be found. The bleeding time was normal. The bone marrow [illegible] and the [illegible] showed a marked increase of megakaryocytes. There was no evidence of fibrosis. In the course of the disease the leucocyte alkaline phosphatase was repeatedly found to be

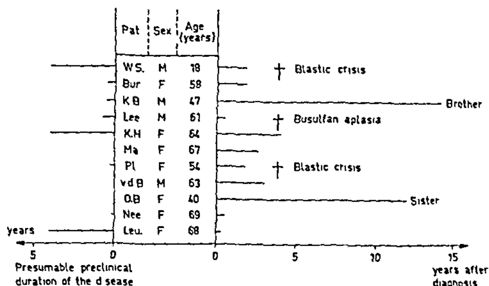


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Cases of Particular Interest

Patient K. R. Caucasian, was first seen in 19[illegible] with [illegible] [illegible] We found [illegible] [illegible] 11,000 leucocytes [illegible]

[illegible] were repeatedly [illegible] could not be found. The [illegible] time was normal. The bone marrow [illegible] and the [illegible] showed a marked increase of megakaryocytes. There was no evidence of [illegible] In the course of the disease the [illegible] was [illegible]

Table I Haematological findings in patient Pl

Date	Hb, g%	Leucocytes/ mm³	Thrombocytes/ mm³	Bleeding time (Ivy), min/sec (N 1–4)	LAP[1] (N 20–80)	Therapy
July 1969	11 6	35,000 (24% basophiles)	3,200,000	2/57	12	Thio-Tepa
January 16, 1970	12.8	8 600	1,200,000	3/10	29	^{32}P
April 17, 1970	14 0	6,300	830,000	–	60	0
July 13, 1970 Blastic crisis	10 2	67,00 (35% blasts) (6% basophiles)	430,000	–	147	Ara-C thioguanine
August 20, 1970 Complete remission	11 3	2,300	700,000	–	–	MTX + Ara C +rubidomycin
October 1, 1970	13 0	5,500	980,000	–	–	MTX + Ara C +rubidomycin
December 16, 1970	14 6(?)	97,000 (80% blasts)	1,270,000	–	–	MTX + Ara-C +rubidomycin
January 6, 1971	11 3	1,900	800,000	–	–	MTX + Ara-C +rubidomycin

[1] Leucocyte alkaline phosphatase index

elevated In 1970, it was 200 Cytogenetics of the bone marrow were performed in May 1972 while the patient was on cytostatic treatment with Thio-Tepa 62 metaphases were normal, 8 showed a hypoploidy.

Treatment consisted of repeated courses of Thio-Tepa intravenously, later busulfan and, temporarily, oral anticoagulation and iron. In October 1972, the patient had a relapse with 970,000/mm³ thrombocytes. This time, he was given 5 mCi ^{32}P

Patient O B The sister of patient K B was found to have hepatosplenomegaly on a routine examination and was, therefore, referred to us She had a hypochromic anaemia with a haemoglobin of 9 6 g%, a normal leucocyte count and 932,000 thrombocytes/mm³ The bleeding time was slightly prolonged The differential blood count showed occasional myelocytes and metamyelocytes Also, many giant platelets were seen The bone marrow biopsy showed an increase of megakaryocytes and a slight increase of reticulin fibres She refused a bone marrow puncture for cytogenic studies Up to 1965, she was treated with intermittent intravenous Thio-Tepa, changing with busulfan After an intermission of 7 years, we saw her again in 1972. She had persistent hepatosplenomegaly Haemoglobin 13 7 g%, leucocytes 4,700 with a normal differential count, platelets 910,000 mm³.



Fig. 2. Case II. Sudan Black B stain of the bone marrow smear with occasional Auer rods in the blast cells. × 800.

Patient P. This [illegible]-year-old woman was first seen in May [illegible] 196[illegible] with fatigue, headaches, easy bruising and at times paraesthesias in the fingers and toes. In June 196[illegible] she had a cerebrovascular accident. On physical examination there was slight hepatosplenomegaly. The laboratory findings are listed in table I. Bone marrow aspiration revealed a severe increase of megakaryocytes. Marrow biopsy and cytogenetic studies were not performed. Treatment was started in a peripheral hospital with [illegible] mg/day [illegible] on 12 subsequent days. This resulted in a decrease of the thrombocytes from [illegible] to [illegible]/mm³. The hematologic course [illegible] between April 196[illegible] and January 197[illegible] she was given a total dose of [illegible] mCi ³²P in 3 to 5 mCi dosages. The platelet counts [illegible] for only very short periods [illegible] a maximum of 2 months. Seven months after the last dose of ³²P she had increasing cutaneous haemorrhages. There was again splenomegaly [illegible] with [illegible]% blasts. Cytochemically, the blasts were markedly positive for Sudan black B. There were occasional Auer rods (fig. 2). Treatment consisted of a combination of cytosine arabinoside [illegible] and [illegible]. She [illegible] complete remission [illegible] and relapsed [illegible] four months after the first complete remission. She had another [illegible] and again [illegible]. A [illegible] remission was reached [illegible]. She died [illegible] months after [illegible]. [illegible]

Patient W. J. He was found to have [illegible] in 196[illegible] at the age of [illegible] years. We first saw him in 197[illegible] at the age of [illegible]. At that time he had a [illegible]

Table I Haematological findings in patient PI

Date	Hb g%	Leucocytes/ mm³	Thrombocytes/ mm³	Bleeding time (Ivy), min sec (N 1–4)	LAP[1] (N 20–80)	Therapy
July 1969	11 6	35 000 (24% basophiles)	3 200 000	2/57	12	Thio-Tepa
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August 20 1970 Complete remission	11 3	2 300	700 000	–	–	MTX + Ara C + rub domycin
October 1 1970	13 0	5 500	950 000	–	–	MTX + Ara C + rub domycin
December 16 1970	14 6(?)	97 000 (80% blasts)	1 270 000	–	–	MTX + Ara-C + rubidomycin
January 6 1971	11 3	1 900	800 000		–	MTX + Ara-C + rubidomycin

[1] Leucocyte alkaline phosphatase index

elevated In 1970 it was 200 Cytogenetics of the bone marrow were performed in May 1972 while the patient was on cytostatic treatment with Thio Tepa 62 meta phases were normal 8 showed a hypoploidy

Treatment consisted of repeated courses of Thio-Tepa intravenously later busul fan and temporarily oral anticoagulation and iron In October 1972 the patient had a relapse with 970 000 mm³ thrombocytes This time he was given 5 mCi ³²P

Patient O B The sister of patient K B was found to have hepatosplenomegaly on a routine examination and was therefore referred to us She had a hypochromic anaemia with a haemoglobin of 9 6 g% a normal leucocyte count and 932 000 thrombocytes/mm³ The bleeding time was slightly prolonged The differential blood count showed occasional myelocytes and metamyelocytes Also many giant platelets were seen The bone marrow biopsy showed an increase of megakaryocytes and a slight increase of reticulin fibres She refused a bone marrow puncture for cytogenic studies Up to 1965 she was treated with intermittent intravenous Thio-Tepa changing with busulfan After an intermission of 7 years we saw her again in 1972 She had persistent hepatosplenomegaly Haemoglobin 13 7 g% leucocytes 4 700 with a normal differential count platelets 910 000 mm³



Fig. 2. Case [illegible]. Sudan Black B stain of the bone marrow smear with occasional Auer rods in the blast cells. × 800.

Patient [illegible]. This 55-year-old woman was first seen in March 1962 with headaches, easy bruising and at times paraesthesias in the fingers and toes. In July 1962 she had a cerebrovascular accident. On physical examination there was slight hepatosplenomegaly. The laboratory findings are listed in table I. Bone marrow aspiration revealed a severe increase of megakaryocytes. Marrow biopsy and cytogenetic studies were not performed. Treatment was started in a peripheral hospital with Thio-Tepa 15 mg intravenously on 12 subsequent days. This resulted in a decrease of the thrombocytes from [illegible] to [illegible]/mm³. The hepatosplenomegaly disappeared. Subsequently, between August 1962 and January 1970 she was given a total dose of [illegible] mCi ^{32}P in [illegible] mCi dosages. The platelets normalized for only very short periods, being a maximum of 2 months. Seven months after the last dose of ^{32}P she had increasing cutaneous haemorrhages. There was again splenomegaly. Leucocytes [illegible]/mm³ with [illegible]% blast cells. Cytochemically the blasts were markedly positive for Sudan Black B. There were occasional Auer rods (fig. 2). Treatment consisted of [illegible] courses of cytosine arabinoside (Ara-C) and 6-thioguanine which was followed by a complete remission. She was maintained with [illegible] (MTX), [illegible] and [illegible]. [illegible] months after the first complete remission she had another relapse with a total of [illegible] leucocytes with [illegible]% blast cells and [illegible] a thrombocytopenia of [illegible]/mm³. A partial remission was reached with a combination of [illegible] and Ara-C. She died [illegible] months later [illegible] from [illegible]. No [illegible] haemorrhage was found at autopsy.

Patient [illegible]. He was found to have hepatosplenomegaly in 19[illegible] at the age of [illegible] years. He was first seen by us in 19[illegible] at the age of [illegible]; at this time he had a [illegible]

duodenal ulcer. Also he was complaining of fatigue, excessive sweating and later paraesthesias of the fingers and toes. There was marked hepatosplenomegaly. Laboratory data are listed in table II. Bone marrow puncture and biopsy showed a very marked increase of megakaryocytes. A liver biopsy showed many megakaryocytes in the sinuses. Later an exploratory laparotomy was performed. The liver was found to be markedly enlarged and, in addition, there was ectopic liver tissue in the retroperitoneal space. Histologic changes were most likely to be due to obstruction in the blood circulation of the liver. He was treated with repeated courses of Thio-Tepa and later intermittent busulfan. The longest remission lasted 2 months. The hepatosplenomegaly decreased only slightly.

In early 1972, he developed increasing leucopenia and thrombocytopenia. Bone marrow aspiration showed a marked infiltration with blasts with occasional Auer rods (fig. 3). Only few blasts were found in the peripheral blood. Cytogenetic examination of the bone marrow in this period was normal. Treatment was started with vincristine and prednisone, later Ara C. The patient died 5 months after blastic transformation without ever having reached a complete remission. Autopsy was refused by the parents. Clinically, the cause of death was septicaemia and cerebral haemorrhage.

Discussion

Chronic thrombocytosis is frequently seen with malignant disease, in particular if located below the diaphragm, and also with rheumatoid arthritis [9]. Myeloproliferative diseases such as polycythaemia vera, chronic myeloid leucaemia (CML) and myelofibrosis are often associated with an increase of thrombocytes. Primary thrombocythaemia is a clinical entity within the chronic myeloproliferative syndromes with a persistent elevation of the platelets above 800 000/mm³ in the presence of a functioning spleen. Characteristic are recurrent haemorrhagic episodes (70–80%) which most frequently occur in the gastrointestinal tract. Most patients have splenomegaly (80–90%), hepatomegaly occurs less frequently (30–50%). Marked thrombocytosis is always found, 80–100% of the patients also have neutrophil leucocytosis and 60–80% have anaemia which usually is hypochromic. Splenic vein thrombosis and atrophy of the spleen are frequent complications. During the frequent paradoxical haemorrhagic episodes, extreme thrombocytosis is the only pathological finding always present. The leucocyte alkaline phosphatase is of little diagnostic value. In our 2 patients with blastic transformation it increased, which is comparable to the situation in CML.

Familial occurrence of myeloproliferative syndromes is rare [15]. To our knowledge, our familial thrombocythaemia cases are the first that are reported.



Fig. 1. Case W. S. Blast cells in the bone marrow smear, one with an Auer rod × [illegible]

Table II. Haematological findings in patient W. S.

Date	Hb g%	Leucocytes mm3	Thrombocytes mm3	Bleeding time (Ivy), minutes (N 1-4)	LAP (N 25-[illegible])	The[illegible]
August [illegible] 1970	[illegible]	23,[illegible]	2,22[illegible],000			[illegible]
September 29, 1970	14.0	13,[illegible] (2% basophilia)	[illegible]	[illegible]	134	[illegible]
June 2[illegible] 1971	15.2	[illegible]	[illegible]		[illegible]	[illegible]
February 23, 1972 [illegible]	12.0	[illegible]	[illegible]	[illegible]	[illegible] [illegible]	[illegible]
May [illegible] 1972	[illegible]	[illegible]	[illegible]	[illegible] [illegible]		[illegible]
[illegible] 1972	[illegible]	[illegible]	[illegible]			[illegible]

That 2 of our patients developed blastic transformation after 12 and 16 months, respectively, from diagnosis, emphasizes that the course of the disease is not necessarily benign One patient was treated with cytostatics only The other had cytostatic agents and ^{32}P To our knowledge, blastic transformation has been described 3 times in primary thrombocythaemia [10, 11, 13], 2 further cases are dubious [1, 12] The first 3 patients [10, 11, 13] all were treated with ^{32}P and the interval between diagnosis and blastic transformation was 22 and 20 months and 5 5 years Blastic transformation is a different condition from acute megakaryocytic leucaemia which takes an acute course to start with and which is not preceded by severe thrombocytosis, bleeding episodes, splenomegaly and thromboembolic complications [2] We have not seen Auer rods in the blasts of blastic crisis of CML, but in both patients with blastic crisis of thrombocythaemia *It is generally accepted that termination in blastic crisis belongs* to the natural history of all chronic myeloproliferative diseases However, it has to be borne in mind that all 5 reported cases including our 2 that made this transition, were treated with ionizing radiation and/or cytostatic agents which may have induced, or at least precipitated, blastic transformation Many authors consider ^{32}P as the drug of choice in the treatment of primary thrombocythaemia [10, 13, 14] In our experience, thromboembolic complications frequently occurred with thrombocytes of 500,000–1,000,000/mm³ and, therefore, we consider anticoagulation essential With platelets in excess of 1 million/mm³, we think that fast-acting alkylating agents are the treatment of choice The correlation between symptoms such as paraesthesias of the fingers and the toes and the number of circulating thrombocytes is poor In selected cases, acetosalicylic acid and other agents interfering with platelet aggregation may be beneficial, but the increased risk of bleeding must be taken into consideration [16]

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The Influence of Repeated and Prolonged Stimulation on the PHA-Response of Lymphocytes in Hodgkin's Disease

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Abstract By repeated and prolonged stimulation the PHA response of lymphocytes in Hodgkin's disease can be modified

Key Words
Hodgkin's disease
Lymphocyte response on PHA stimulation

The decreased lymphocyte response to PHA stimulation is a well-known feature of the immunologic disorder of Hodgkin's disease [1–7] A prolonged stimulation could elicit an increase in lymphocyte response as well as it occurs in chronic lymphocytic leukaemia [8–11] Moreover, an increase could also be observed using a repeated stimulation with small quantities of PHA The results obtained with these different kinds of stimulation in Hodgkin's disease lymphocytes are reported in this paper

Materials and Methods

Blood was collected from 20 patients with active Hodgkin's disease, 8 patients were at stage II 10 at stage III and 2 at stage IV Blood lymphocytes were separated by Ficoll Hypaque centrifugation [12] The lymphocytes were washed three times with Hanks solution and then resuspended in 199 TC Medium (Difco Lab Detroit Mich) at the concentration of 1×10^6 cells/ml 20% of autologous serum was added

Four lymphocyte cultures were prepared for each patient Each culture was stimulated in different kinds. The first group was added to the mitogen PHA dose (0 2 ml PHA M Difco Lab Detroit Mich 5 ml of sterile distilled water per bottle) at the start and the cultures were stopped at 72 h The second group stimulated like the first one was stopped at 144 h One eighth three eighths and one half of the

Table I. Clinical features of patients with Hodgkin's disease

Case No.	Age	Sex	Stage	Therapy
1	35	M	IIIa	not treated
2	41	M	IIIa	irradiation (18 months prior to study)
3	33	F	IIIa	not treated
4	42	F	IIIa	not treated
5	29	F	IIIa	not treated
6	30	M	IIIb	not treated
7	43	M	IIIa	not treated
8	24	F	IIb	irradiation (1 year prior to study)
9	18	M	IIb	not treated
10	25	F	IIa	not treated
11	44	F	IIa	not treated
12	30	F	IIa	not treated
13	32	M	IIa	not treated
14	50	F	IIb	not treated
15	21	M	IIa	not treated
16	27	M	IIIa	not treated
17	42	F	IIIb	not treated
18	31	M	IVb	vinblastine, prednisone, procarbazine (15 months prior to study)
19	29	M	IVa	not treated
20	22	M	IIIb	not treated

mitogen PHA dose were added to the third group at the start and at 12 and 24 h, respectively; the cultures were stopped at 72 h. The fourth group of cultures, stimulated like the third one, was added to a whole PHA mitogen dose at 72 h, and the culture time was prolonged till 144 h. Each group was evaluated for blastic transformation on a morphological basis.

Results

The average results obtained in each group of cultures are reported in table II. In the first two groups (a and b) treated by standard PHA stimulation, a significant increase of lymphocyte response is observed, prolonging the incubation culture time ($p<0.01$). A very significant difference ($p<0.001$) is also observed between the cultures treated by standard stimulation for 72 h (group a) and 144 h (group b) and the 144 hour cultures stimulated by repeated small PHA doses and finally added to a mitogenic dose (group d).

Table II. Lymphocyte transformation: mean, standard deviation and statistical comparison (Student t for paired data)

	a	*b*	
No. of cases	20	20	
Mean	39.75[2]	47.65[1]	$p<0.01$
SD	7.04	9.81	
	c	*d*	
No. of cases	20	20	
Mean	45.85[1]	53.25[2]	$p<0.05$
SD	10.33	10.00	
	$p<0.01$	$p<0.001$	

[1] $p>0.05$
[2] $p<0.001$

The use of double PHA doses is not important as demonstrated by results of the group c and d cultures; there is no significant difference between these two groups of cultures ($p<0.05$), even if these data are certainly influenced by the different culture times (72 h for group c and 144 h for group d).

The influence of repeated small stimulations is also confirmed by the results obtained with the group c cultures: the lymphocyte response in this group is significantly increased as compared with the group a cultures ($p<0.01$). No significant difference is observed between group b and c cultures ($p>0.05$).

Discussion

Our results demonstrate that the decreased lymphocyte response to PHA stimulation in Hodgkin's disease can be modified by both prolonging the culture time and using a different kind of stimulation. The influence of culture time can be attributed to a time-dependent impairment of lymphocyte response, as if some steps in the response would be more time-consuming. With the particular kind of stimulation we used, the activity of mitogen is highly favoured, perhaps modifying the interaction between mitogen and membrane receptors. We observed that in Hodgkin's

disease the lymphocytes can respond to mitogen almost as normal lymphocytes when using some particular kind of stimulation. In these experimental conditions an 'obstacle' probably present in lymphocyte membrane can be removed or overcome.

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Enhanced Uptake of Anti-Rh_0 Coated Red Cells by Cultured Human Monocytes

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Abstract Red cells coated with anti Rh_0 antibody (IgG) were extensively ingested by human blood monocytes and macrophages and only rarely by neutrophils The erythrophagocytosis was inhibited by free IgG_1 and IgG_3 Less inhibition occurred with IgG_2 and IgG_4 indicating that the mononuclear receptor was specific for IgG_1 and IgG_3 During cell culture, the ability of monocytes to ingest anti Rh_0 coated red cells increased to the degree of that of pleural and peritoneal macrophages

Key Words
Erythrophagocytosis
IgG receptor
Macrophages
Monocytes
Monocyte culture

The origin and kinetics of proliferation of monocytes and macrophages have been investigated for many years [6] Recently, based on studies performed mainly with radioisotopes, the nomenclature of 'Mononuclear Phagocyte System' [11] has been proposed to delineate the monocyte cell series originating from bone marrow through blood to macrophages in various tissues

The presence in human monocytes and macrophages of the IgG receptor for red cell IgG antibody complexes has been demonstrated [1–4, 7–10, 12, 13], and some investigators claimed that the receptor may be used as an immunological marker for the characterization of mononuclear cells

This paper reports the functional properties of blood monocytes in relation to macrophages with IgG receptor as a marker

Materials and Methods

Antisera Anti Rh_0 (anti D) serum which had an indirect Coombs titer of 1 256 was obtained from Ortho Pharmaceutical Corp (USA) The antiserum consisted

marily of IgG ... A and IgM constituted less than 5 and 1% of total globu... 1 ex. respective ... rmal IgG was obtained from Midori Juji Co. (Japan). IgG subclasse ... G_4 purified from heavy chain disease of each subclass and IgG_4 ar ... na proteins were kindly supplied by Dr. K. Takatsuki. IgA and m ... nples were purified from the sera of patients with IgA myeloma ... a macroglobulinemia, respectively.

Red ... Human type O red cells washed 3 times in Hanks solution were ... h anti-Rh, antibodies by incubating for 1 h at 37 °C, af... ter wa... washed in Hanks solution.

As ... cytes. Normal human white cells, leukemic leukocytes of various ... al and peritoneal macrophages, adjusted to 1.2 × 10⁶ ml were ... 1 x ... tissue culture chamber slides (Lab-Tek Products, Division Mi... ... ... USA). Sensitized red cells were placed over the mono-layer ... a 0.5 percent suspension, and incubated for 1 h at 37 °C ... nonadh... red ... ere then removed by several washes with Hanks solution. Phago... ... experiments were performed by adding the test protein to the m... ... addition of red cells coated with anti-Rh_o.

Ce ... ... lear cells from patients with monocytic leukemia were suspend... ... 20% calf serum and antibiotics, and placed in Lab-Tek tissue c... ... rs. They were gassed with 5% CO_2, 95% air and incubated for ... ... 3 days. The cells were washed three times with Hanks solution ... ... the presence of the IgG receptor.

Results

Inte... ... ates with *IgG-sensitized red cells and its inhibition by* ... After ... h incubation at 37 °C, nearly 100% mature monocytes ... IgG-se... red red cells, while 1.5% of neutrophils ingested erythro... ... Phagocytosis of red cells by mature monocytes isolated from monoc... leukemia ... urred at a rate similar to that observed with normal m... ... red cell ingestion by immature cells (blasts and promonocytes) ... observed.

The ... phagocytosis by monocytes was strongly inhibited by low ... concentration of IgG ... the incubation medium. At the concentration of 5 g/m... ... IgG_1 and IgG_3 showed strong inhibitory effect on the ... ... red cells by monocytes. Other IgG subclasses, IgG ... and IgA, IgM and IgD did not affect the erythrophago-cytosis ...

Incubation ... ... antibody at 56 °C for 30 min ... which red ... did not affect the uptake of red cells by monocytes ...

Effe... ... on the phagocytosis of IgG-coated red



Fig 1 Uptake of anti Rh coated red cells by blood monocytes cultured monocytes and macrophages *a* Human leukocytes from monocytic leukemia incubated with anti Rh_o coated red cells Monocytes containing ingested red cells negative neutrophils *b* Monocytes which had been cultured for 3 days, then incubated with anti Rh_o-coated red cells Monocytes containing a large number of ingested red cells are seen *c* Pleural macrophages incubated with anti Rh_o-coated red cells

cells Blood monocytes isolated from peripheral blood of monocytic leukemia were cultured in Lab-Tek tissue culture chamber slides The number of ingested red cells per mononuclear cells increased markedly in the course of cell culture from a mean of 2 cells at the start of cell culture to

9 cells on the 3rd day of the experiment, as shown in figure 1. It also shows that pleural and peritoneal macrophages which exhibited enhanced phagocytic activity with more than 10 ingested erythrocytes per cell. A 10- to 100-fold higher concentration of IgG was necessary to inhibit the red cell uptake by these cultured monocytes and by pleural and peritoneal macrophages than was needed to affect the erythrophagocytosis by blood monocytes.

Discussion

Recently CLINE and SUMNER [5] reported functional characteristics of mononuclear phagocytes in mouse bone marrow in terms of ability to adhere to glass, phagocytosis and surface receptor for immunoglobulin. HUBER *et al.* [1] described surface receptors for IgG in blood monocytes as well as in spleen and liver macrophages.

We examined functional ability of human blood monocytes with regard to cell surface receptor for IgG. Immature monocytes obtained from monocytic leukemia showed low phagocytic activity for IgG-sensitized red cells. Mature monocytes exhibited a progressively increased ability of becoming erythrophagocytic in the course of cell culture. This ability equalled that of pleural and peritoneal macrophages. The enhancement of monocyte function during cell culture appears to be due to an increased number of monocyte receptors, since a much higher concentration of IgG was necessary to inhibit erythrocyte ingestion by cultured monocytes than when blood monocytes were incubated. Subclass specificity of mononuclear receptor for IgG_1 and IgG_3 was confirmed by our study.

With lysozyme (muramidase) as a marker, steady release of the enzyme from skin window mononuclear cells has been demonstrated previously [14, 15]. Lysozyme negative mononuclear cells predominated 50 h after the onset of skin window study and such an early loss of lysozyme from monocytes may support the cellular conversion between blood monocytes and macrophages in other sites such as bone marrow, pleural and peritoneal cavity which usually lack the enzyme. Cell culture of monocytes resulted in lysozyme-negative cells comprising the majority of such cells after 3 days culture. Thus, ability to ingest anti-Rh-coated red cells increased during monocyte culture to the degree of that shown by pleural and peritoneal macrophages, while lysozyme activity decreased to a lower degree. The results may indicate a close relationship between blood monocytes and tissue macrophages. However, further studies have to be ca-

une fuite plasmatique, celle-ci expliquerait l hémoconcentration observée lors d'accidents de décompression

Les agrégats de plaquettes et d hematies ont été mis en évidence par PHILIP *et al* [9] dans la circulation pulmonaire après des accidents de décompression sévères chez le rat, et sont semblables à ceux obtenus lors de coagulations intravasculaires disséminées PHILIP *et al* [10] ont montré que chez l homme, même en l'absence d accidents de décompression, il y avait une diminution de 10 à 12% du nombre des plaquettes circulantes MARTIN et NICHOLS [6] ont trouvé que la baisse du nombre des plaquettes est maximale 3 jours après une plongée expérimentale à 4 aTA, sans symptômes

Il nous paru intéressant de mettre en évidence la présence éventuelle des agrégats plaquettaires circulants par la technique de la pression de filtration [12] et de confirmer la chute du taux des plaquettes circulantes au cours d'accidents de décompression chez le rat Dans ce même travail, nous avons également étudié les modifications de l'agrégabilité des plaquettes, dans les mêmes conditions, dans le but d expliquer la survenue d'agrégats

Méthodes

Mesure de la pression de filtration et comptage des plaquettes après décompression rapide Nous avons utilisé un lot de 80 rats mâles de 375 à 450 g élevés avec nourriture et eau à volonté Pour obtenir les accidents de décompression nous avons comprimé à l air dans un petit caisson les rats par groupe de deux, à la vitesse de 1 atm/min pour atteindre la pression de 8 aTA Après 1 h la décompression est réalisée à la vitesse de 1 atm/20 sec. Le sang est recueilli par section rapide d une carotide repérée par un fil un jour avant.

Les animaux sont groupés en quatre lots Le premier est sacrifié sans avoir été comprimé (28 rats) et constitue le lot témoin, le deuxième comprend des rats sacrifiés 3 min après décompression (31 rats), le troisième lot comprend tous les rats ayant présenté un accident de décompression entre la 3e et la 30e min et immédiatement sacrifiés (23 rats) enfin les rats ne présentant pas d accidents dans ce laps de temps sont tous sacrifiés à la 30e min (15 rats) et constituent le 4e lot.

Le sang de chaque animal est recueilli en flacon plastique sur du citrate de sodium à 9‰ (2 ml de citrate pour 10 ml de sang) Le sang citraté est centrifugé pendant 10 min pour obtenir du plasma riche en plaquettes (PRP) Sur le PRP, on fait une numération plaquettaire et une mesure de la pression de filtration

La numération des plaquettes est faite au microscope et toujours par le même expérimentateur après dilution au 1/100 avec de l oxalate d ammonium. Pour le PRP d animaux ayant fait un accident de décompression nous avons vu dans le champ quelques agrégats plaquettaires pour lesquels on ne peut compter le nombre des plaquettes, étant donné la métamorphose visqueuse

La mesure de la pression de filtration [11] est dérivée de la technique de Swank [12]. Elle consiste à mesurer la pression d'une suspension (en l'occurrence le PRP) lorsqu'on la fait passer, avec un débit constant, à travers un filtre parfaitement calibré à pores cubiques de 20 µm d'arête. La pression mesurée par un capteur de pression est exprimée en mm Hg. D'éventuels agrégats plaquettaires vont alors obstruer le filtre et provoquer une augmentation de pression en amont.

Mesure de l'agrégation des plaquettes. Les accidents de décompression sont provoqués par la même technique que précédemment, le sang est prélevé par ponction cardiaque sous anesthésie générale légère.

A partir du sang de chaque animal, on prépare du PRP par centrifugation et du plasma pauvre en plaquettes (PPP) par centrifugation à 6000 rpm pendant 20 min. On compte alors les plaquettes des PRP que l'on ajuste entre 200 et 300 $\times 10^3$ plaquettes par mm³ avec du PPP. Les mesures ont été faites chez 15 rats témoins et chez 14 rats ayant subi une décompression rapide.

L'agent agrégant a toujours été l'ADP à des concentrations finales de $2,4 \times 10^{-4}$, $2,4 \times 10^{-5}$ et $2,4 \times 10^{-6}$ M. L'appareil utilisé est du type «Mustardo» et l'enregistrement a toujours été réalisé pendant 10 min. L'appareil est réglé sur 0 % pour le PRP et sur 100 % pour le PPP.

Les paramètres retenus pour chaque dose étudiée ont été :

Hauteur maximale relative

$$H_{max r} = \frac{H_{max}}{H\ 100\ \%}$$

Il s'agit de l'extremum atteint entre la ligne de base et la courbe de l'enregistrement photométrique.

Hauteur minimale relative

$$H_{min r} = \frac{H_{min}}{H\ 100\ \%}$$

La définition est identique à celle du Hmax.

[illegible] à 45 sec [illegible]. Ce paramètre correspond le plus souvent à la pente de la tangente d'inflexion.

Aire relative d'agrégation

$$A_r = \frac{\text{aire d'agrégation}}{\text{aire 100 \%}}$$

Ce paramètre a été [illegible] pour toutes nos mesures à l'aide d'un intégrateur [illegible]. Il correspond, pour un temps donné, au rapport de l'aire comprise entre la ligne de base et la courbe d'agrégation rapportée à l'aire comprise entre la ligne de base et le PPP.

Résultats

Pression de filtration et numération des plaquettes. Les résultats ont été [illegible] de Student. Le nombre de

Tableau I Influence de la decompression sur la pression de filtration et la numération des plaquettes

	Pression de filtration, mm Hg		Nombre de plaquettes par mm³ de PRP	
Témoins	13,72 ± 1,12	(22)	588,28 × 10³ ± 43 59 × 10³	(28)
Décomprimés sans accident	13,68 ± 1,23	(35) NS	421,24 × 10³ ± 28,26 × 10³	(41) S
Décomprimés avec accident	20,19 ± 3,17	(16) S	287,83 × 10³ 33 07 × 10³	(23) S

Le nombre d'animaux pour la série est indiqué entre parenthèses, S = significatif à 0,05, NS = non significatif

rats de chaque série pour les deux mesures n'est pas le même, car, pour des raisons techniques, les deux mesures n'ont pas toujours été faites sur le même rat

Dans un premier temps, nous avons comparé les résultats entre les mesures du lot 2 et du lot 4 Cette étude nous a permis de montrer que ces deux populations étaient statistiquement homogènes De plus, étant donné que ces rats ne doivent plus faire d'accidents de décompression, nous avons regroupé nos résultats en trois groupes témoins, rats décomprimés sans accident et rats décomprimés avec accident [3]

On constate alors (tab I) qu'il existe une chute significative du nombre des plaquettes circulantes dans le PRP chez des rats décomprimés sans accident par rapport aux rats témoins (–28%) La diminution est encore plus importante chez les rats ayant présenté un accident (–51%) La pression de filtration ne varie pas chez les rats décomprimés sans accident, il n'existe donc pas d'agrégats circulants dont la taille est supérieure à 20 μm et qui pourraient colmater les pores des filtres utilisés, par contre, elle augmente légèrement chez ceux présentant un accident de décompression

Agrégation des plaquettes (tab II) Pour une concentration en ADP de l'ordre de $2{,}4 \times 10^{-4}$, nous n'observons pas de différence significative entre les deux lots L'agrégation est irréversible dans les deux cas (*$Hmin_r$ et $Hmax_r$ ne different pas*) Cependant, la vélocité de Hmax, (indiquant l'intensite maxima) sont legèrement plus faibles (significatif à 10%) après décompression une legère modification de l'agrégabilité des plaquettes est donc decelable

Tableau II. Influence de la décompression sur l'agrégation des plaquettes à l'ADP

Concentration finale en ADP	Paramètres	Témoins (15 animaux)	Après décompression (14 animaux)	Signification statistique
2,4 × 10[illegible] M	Hmax	82,5 ± 2	77,5 ± 3,1	NS
	Hmax	85,1 ± 1,7	78,2 ± 4,2	NS (5 à 10 %)
	V	41,7 ± 1,6	35,5 ± 3,6	NS (5 à 10 %)
	A_e	[illegible] ± 2,2	70,6 ± 4	NS
2,4 × 10[illegible] M	Hmax	65,2 ± 6,2	51,9 ± 6,1	NS
	Hmax	91,6 ± 2,2	67,6 ± 5	5 %
	V	44,3 ± 2,9	32 ± 2,6	[illegible] ‰
	A_e	43,6 ± 2,5	[illegible] ± 4,7	NS (5 à 10 %)
2,4 × 10[illegible] M	Hmax	31,5 ± 5	19,4 ± 3,9	5 %
	Hmax	64,3 ± 2,6	[illegible] ± 4	1 ‰
	V	41,3 ± 3	26,5 ± 2,7	5 ‰
	A_e	41 ± 4,5	24,9 ± 3,3	2 %

Pour une dose de 2,4 × 10[illegible] M d'ADP, les différences sont plus nettes et sont significatives pour V et Hmax. Par contre, l'aire A ne diminue pas significativement. Par rapport aux témoins, les courbes obtenues pour cette sensibilité chez les rats décomprimés rapidement, seraient plutôt de type irréversible.

Pour la dose de 2,4 × 10[illegible], tous les paramètres sont inférieurs (de façon statistiquement significative) : il existe chez les rats décomprimés rapidement une diminution de la vélocité, du maximum d'agrégation et de l'intensité globale de la réaction traduisant une hypoagrégabilité probable des plaquettes.

Discussion

En ce qui concerne la numération des plaquettes, nos résultats confirment ceux obtenus sur l'homme par Philp et al. [11] et Martin et Nichols [8]. La chute du taux des plaquettes est plus importante quand la décompression est [illegible] beaucoup plus rapide dans notre expérimentation. Il y a donc bien des agrégats plaquettaires dès la décompression, mais en fait ceux-ci sont de faible taille [illegible] et la pression de filtration ne change pas. Il [illegible] important que lorsqu'apparaissent des symptômes d'accident de décompression et la pression de filtration augmente alors.

Il est important de savoir dans quelle région ces agrégats sont éventuellement séquestrés Des scintigraphies après injection de plaquettes marquées au chrome radioactif, ont été faites sur des rats témoins et sur des rats décomprimés l'étude est en cours, mais on peut d'ores et déjà dire que la répartition des plaquettes, homogène chez les rats témoins, est très différente chez les rats décomprimés ou les plaquettes semblent surtout localisées au niveau du foie, de la rate, et également des poumons

D'autre part, les résultats d'agrégation montrent qu'il y a hypoagrégabilité des plaquettes circulantes recueillies chez les rats après décompression rapide On pourrait alors penser qu'il y a contradiction avec le fait qu'il y ait simultanément chute du nombre des plaquettes circulantes et existence de micro-agrégats disséminés qui sont bien plutôt le signe d'une hyper agrégabilité

C'est certainement le cas et l'on peut supposer que les plaquettes sanguines les plus jeunes, donc les plus réactives, ont pu s'agréger localement tandis que les plaquettes les plus vieilles, donc moins réactives, seraient en plus grand nombre dans le sang circulant et donc dans le sang que nous avons pu prélever après accident Seule l'étude (que nous avons entreprise), de la répartition des populations plaquettaires avant et après décompression rapide pourra nous expliquer cette discordance apparente Remarquons seulement que nos premiers résultats sur 4 rats ayant subi une décompression semblent confirmer une telle hypothèse

Résumé

Les auteurs étudient les modifications des plaquettes sanguines du rat au cours des accidents de décompression Les résultats montrent une augmentation de la pression de filtration lors de l accident alors qu il faut noter une réduction très importante du nombre de plaquettes Une telle constatation est certainement la manifestation d agrégats plaquettaires disséminés dont une faible partie seulement est circulante D'un autre côté, la mesure de l agrégation photométrique montre l apparition d une hypoagrégation plaquettaire que l on peut attribuer à une modification de la répartition des populations plaquettaires.

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Lysozyme-Negative, Peroxidase-Positive Mononuclear Cells: a New Kinetically Distinct Cell Population in Normal Rat Blood

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With 1 color plate

Abstract Lysozyme negative, peroxidase-positive (L–P+) cells are small mononuclear cells which were found to constitute 3.4±1.3% (mean±SD) of the leukocytes in normal rat blood When tritiated thymidine was injected into rats at 6-hour intervals for 5.5 days a maturation time of 3–4 days preceded the appearance of the first labelled L–,P+ cells in the blood Unlabelled and heavily labelled L–,P+ cells left the circulation at random with a half time of 28–31 h Both the percentage of labelled cells and the grain count data showed that the L–,P+ cells are a uniform cell population differing kinetically from all previously known blood cell populations

Key Words
Autoradiography
Cell kinetics
Cytochemistry
DNA synthesis
Lysozyme in blood cells
Monocytes
Peroxydase staining
Rat blood

In an investigation of the usefulness of 2 cytochemical methods for the identification of monocytes [6], a staining method was developed that allowed the simultaneous demonstration of lysozyme and peroxidase activities in the same cell When normal rat blood cells stained by this method were examined, attention was drawn to the distinct cytochemical pattern exhibited by a significant proportion of small mononuclear cells These cells showed a clearly visible peroxidase reaction, but no or only very slight lysis of the bacteria constituting the substrate of lysozyme Typical monocytes showed both a peroxidase reaction and a broad zone of lysed bacteria, and typical lymphocytes showed neither reaction The existence of lymphocyte like cells showing a positive peroxidase reaction by the staining technique of RYTÖMAA [2] has previously been suggested by the results of WHITELAW [7], who defined rat blood monocytes as peroxidase-positive mononuclear cells

This article reports the number, morphology, and labelling characteristics of these lysozyme negative, peroxidase positive (L−P+) cells in an experiment where frequent intermittent injections of 3H thymidine were given to rats. The simultaneous labelling of the lysozyme positive mononuclear blood cells (monocytes) in the same rats has previously been reported [5].

Material and Methods

[illegible] rats were given i.p. injections of 0.2[illegible] µCi/g of 3H thymidine at 6 hour intervals for [illegible] days, and mixtures of [illegible] blood and a suspension of [illegible] *M. [illegible]* [illegible] bacteria were smeared and stained by a combined method for lysozyme and peroxidase [illegible]. The smears were coated with autoradiographic emulsion (Kodak NTB[illegible]), exposed for [illegible] days, developed, and counterstained as previously described [5]. The autoradiographic background in the control preparations stained by the combined method did not differ significantly from that in the preparations stained for lysozyme, and thus the pooled values, separately calculated for each batch of [illegible] smears, were used both in this and in the other part [5] of the experiment for background correction by the method of [illegible] [illegible].

Definition of 2 groups of L−P+ cells. The L−P+ cells were classified in 2 groups: those with no detectable lysozyme activity (group 1) and those with a trace of activity [illegible] of morphological changes in [illegible] bacteria immediately [illegible] the cell (group 2). It is seen in figures 1a, b that the bacterial changes around the L−P+ cells of group 2 were [illegible] as compared to the broad zone of [illegible] bacteria surrounding the monocytes and neutrophils.

To avoid errors caused by variable staining of different preparations or parts of preparations, no cell was considered lysozyme negative before it had been ascertained that the nearest neutrophil or monocyte was distinctly positive.

Counting methods. The number of L−P+ cells was determined by examining [illegible] leukocytes per rat in the preparation of each one of the [illegible] days on which the [illegible] [illegible].

The grain count of [illegible] L−P+ cells of group 1 and [illegible] L−P+ cells of group 2 were recorded per rat for each one of the 12 time points studied. The majority of these cells [illegible] [illegible] of [illegible] smears in order to find [illegible] L−P+ cells.

The grain counts of [illegible] [illegible] per rat were recorded to get the percentage of labelled neutrophils [illegible] of [illegible] days.

Results

Number of L−P+ cells. The L−P+ cells [illegible] [illegible] [illegible] of [illegible] [illegible] [illegible] of blood [illegible].

[illegible] the [illegible] and [illegible] number of L−P+ cells [illegible]

rise with time in all rats By a two-tailed Student's t test this trend proved significant in 3 rats The mean values for all rats showed a significantly rising trend ($p \approx 0.01$ for absolute cell counts and $p < 0.01$ for percent values) Analysis of variance (one factor design) revealed significant differences between the values for the 9 days ($F = 3.16$ for absolute cell counts, $F = 3.77$ for percent values) The total leukocyte counts of the rats on the same 9 days, which have previously been reported [5], remained unchanged during the experiment

Of the 921 L–,P+ cells counted, 34% were classified as group 1 cells and 66% as group 2 cells

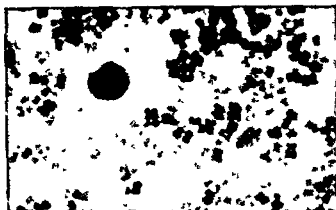
Morphology of L–,P+ cells Because a variable degree of shrinking was observed in the cells of different preparations and parts of preparations, the size of the L–,P+ cells was only crudely estimated by visually comparing the cells with the nearest lymphocytes and monocytes Analysis of 600 L–,P+ cells on day 14 gave 43% cells of the size of small lymphocytes, 55% cells of the size of large lymphocytes and 2% cells of the size of monocytes

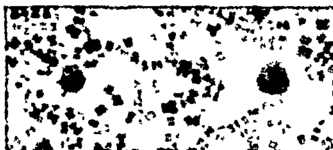
The cell morphology was not preserved well enough to permit observation of morphological details However, as can be seen in figure 1, the L–,P+ cells were small mononuclear cells, often closely resembling lymphocytes Part of the larger L–,P+ cells, like the cell seen in figure 1a, resembled morphologically the monocytoid cells previously described [6]

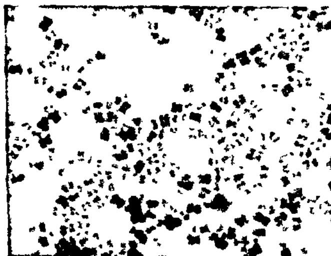
Labelling percentage and grain count of the 2 groups of L–,P+ cells The mean percentage of cells labelled at each time point studied was plotted separately for group 1 and group 2 cells The curves were similar in shape, but there was a small time interval between their rising parts that was constant and therefore probably significant The first labelled L–,P+ cells appeared in the blood after a period of 3 days for group 2 cells and 4 days for group 1 cells Both groups of cells attained the maximum labelling index (97%) on day 11

The mean grain count per labelled L–,P+ cell (mean values of all rats) at each time point studied was also plotted separately for group 1 and group 2 cells The curves were similar in shape, but again a small time difference corresponding to that of the curves showing labelling percentage was seen

Because the labelling of group 1 and group 2 cells was thus very similar, the results obtained separately for the 2 groups were combined in







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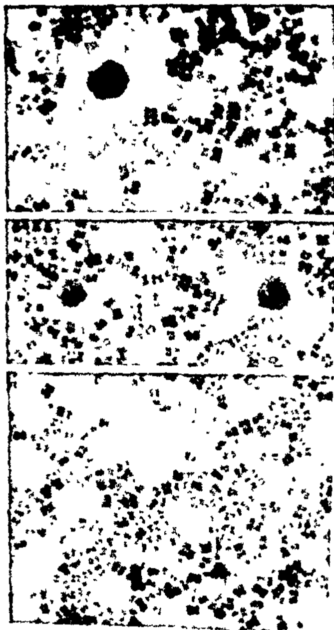


Fig. 1. L-P+ cells and other cells of rat blood in autoradiograms stained for the demonstration of lysozyme and peroxidase activity. A broad zone of lysed *Micrococcus lysodeikticus* bacteria is seen around a monocyte (a, left) and a neutrophil (b, right). The 2 lymphocytes (c, left) show neither the yellow peroxidase reaction nor morphological changes in the bacteria surrounding the cells. The L-P+ cells show the peroxidase reaction but no (c) or only a few (a, b) lysed bacteria around the cells. × 1 600

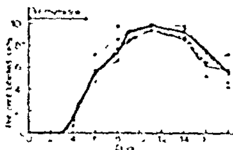


Fig. 2. Percentage of L−P+ cells labelled in the blood of 6 rats during and after intermittent injections of ^{3}H thymidine

order to increase the material available for estimation of the values for the whole population of L−P+ cells. Although the number of group 1 and group 2 cells in the blood was not exactly the same, 50 + 50 cells per rat and time point were considered representative for the whole population.

Percentage of labelled L−P+ cells. At one hour after the first injection of ^{3}H thymidine the method of calculation used [3] gave 13.4% labelled L−P+ cells. However, on days 1 and 2 it gave 0% labelling. As it seems logically impossible that L−P+ cells could be labelled at one hour, but not on days 1 and 2, the 13.4 percent labelling observed must be 'false' and due to the presence of more background in the test preparations than in the controls of this particular batch. That background grains are not always distributed at random over test and control cells has been reported by [illegible] [4]. Thus the true labelling index of L−P+ cells at one hour was zero. Furthermore, the grain count distribution of L−P+ cells did not differ significantly from the average background distribution in the whole experiment, and none of the L−P+ cells showed more than 5 grains.

The percentage of L−P+ cells labelled in the individual rats at the various time points studied is shown in Fig. 2. [illegible] the theoretical labelling percentage on day [illegible] [illegible] that there were a few faintly labelled L−P+ cells (of group 2) on

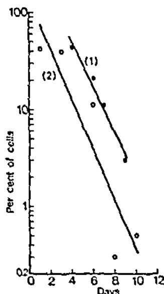


Fig 3 Disappearance from the blood of unlabelled L-,P+ cells (●) on days 6-11 and of heavily labelled (11 or more grains) L-,P+ cells (○) on days 9-18 when intermittent injections of ³H thymidine were given on days 1-5 Regression line (1) estimating equation log Y = 1 94 – 0 2333 X, standard error of estimate 0 09 Regression line (2) estimating equation log Y = 2 14 – 0 2591 X, standard error of estimate 0 48

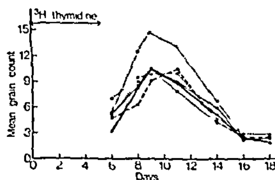


Fig 4 Mean grain count per labelled L-,P+ cell in the blood of 6 rats during and after intermittent injections of ³H thymidine

that day. It may be noted that maximum labelling was attained after a time lapse of 5.5 days from the cessation of ³H thymidine injections.

The mean percentage of unlabelled L-P+ cells decreased exponentially between days 6 and 11, as is shown by the linear decline in a semilogarithmic plot (fig. 3). Although the results would not have been essentially changed, the value on day 4 was not used in the calculations because of its probable inaccuracy. The half time given by the estimating equation was 31.0 h. The turnover time of blood L-P+ cells calculated from this value is 44.6 h. The mean percentage of heavily labelled cells, defined as cells with 11 or more grains, also showed an exponential decline after a maximum on day 9 (fig. 3). Calculations gave a half time of 27.9 h.

Grain count of L-P+ cells. The mean grain count per labelled L-P+ cell in the individual rats at the various time points studied is shown in figure 4. As the estimation of the mean grain count per labelled cell is inaccurate when only a few labelled cells are present, the probably too low values (range 0.8–2.8 grains per cell) on days 3 and 4 were not taken into account. In each rat the mean grain count thus rose from the initial value on day 6 to a maximum that was approximately twice this value. The mean value for all rats rose from 5.1 (day 6) to 10.9 (day 9). Between days 11 and 16 the mean value for all rats declined exponentially (estimating equation: log Y = 1.13–0.1175 X; standard error of estimate 0.06) with a half time of 2.6 days (61.7 h).

Percentage of labelled neutrophils. The mean percentage of blood neutrophils labelled was 2.0% on day 2, 65% on day 3 and 93% on day 4.

Discussion

The absence of labelled L-P+ cells in the blood after the first injections of ³H thymidine shows that circulating L-P+ cells do not synthesize DNA. There was a time lapse of 3–4 days before the first labelled L-P+ cells appeared in the blood after the start of labelling. The peroxidase L-P+ cells thus take at their place of origin, presumably in [illegible], [illegible] a process of maturation before being released into the blood. The first cells to appear were those showing a trace of [illegible] activity (grain 2 cells). These cells were probably more differentiated than the [illegible] and [illegible].

The disappearance of unlabelled L-,P+ cells from the blood was exponential, indicating that the cells leave the circulation at random The disappearance rate gives a direct measure of the turnover time of blood L-,P+ cells, provided that the cells are released from the pool of maturing cells according to the pipe-line principle and there is no compartment from which recirculation of mature L-,P+ cells takes place If the above assumptions do not hold, the value obtained (45 h) represents an overestimation of the turnover time

A model of the L-,P+ cell system involving only one precursor cell with stem cell characteristics is consistent with the finding that the maximum mean grain count was twice the initial value Another model consistent with the results is one with several succeeding dividing precursor cells, each incorporating the same amount of label per phase of DNA synthesis The half-time of the mean grain count of labelled blood L-,P+ cells (2 6 days) gives an upper limit of the average generation time of the precursor(s)

It is thus clear that the L-,P+ cells differ from the monocytes in the blood of the same animals not only with regard to morphological and cytochemical characteristics, but also with regard to their kinetics If the curves showing the percentage of labelled monocytes [5] and L-,P+ cells as a function of time are combined in such a way that the 7 3 ratio of monocytes to L-,P+ cells in the blood is taken into account, the resulting curve is very similar to the curve obtained by WHITELAW [7] for rat blood monocytes

Degenerated neutrophils sometimes surprisingly resemble lymphocytes in appearance [1], and it might be suggested that the L-,P+ cells were neutrophils that had lost their lysozyme activity and characteristic morphological appearance The labelling of neutrophils was, however, markedly different from that of the L-,P+ cells and, as can be seen in figure 1b, the neutrophils were in fact relatively well preserved

This investigation shows that the L-,P+ cells are a uniform cell population with cytochemical and kinetic characteristics that differentiate them from all previously known blood cell populations It can be suggested that they are a subpopulation of lymphocytes or belong to the group of mononuclear phagocytes, but their proper classification is not yet possible

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Splenic Feedback in Red Cell Regeneration

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Key Words
Erythrophagocytosis
Erythropoiesis
Hemolysis
Splenic feedback

Abstract Equivalent hemolysis was produced in adult albino rats by intracardiac injection of copper sulfate phenylhydrazine and antiserum Reticulocyte response and hemoglobin formation were studied in relation to splenic erythrophagocytic activity, hepatic erythrophagocytosis and evidence of *intravascular hemolysis* *Splenic* feedback produced an early, rapid reticulocyte response when accompanied by a fall in the hemoglobin levels whereas hepatic erythrophagocytosis and intravascular breakdown produced a slower response

The spleen is an important destruction site for effete and abnormal red cells It is likely that the red cell levels are maintained by feedback mechanisms from this organ With this in view, splenic activity was studied using different hemolytic agents

Materials and Methods

Adult albino rats of either sex weighing 150 250 g were used in all experiments The doses of three different hemolytic agents were adjusted to produce approximately 3 mg% of serum bilirubin after 24 h as a sublethal dose of copper sulfate produced this degree of hemolysis The agents used were copper sulfate (AR Grade 5 000 μg 200 g body weight) phenyl hydrazine (20 mg/200 g body weight) and anti rat erythrocyte serum raised in rabbits (1/10 dilution 1 ml/200 g body weight) The animals were sacrificed in batches of nine rats each at intervals of 1 2 3 5 and 7 days

The serum bilirubin levels reticulocyte counts and hemoglobin levels were determined before injecting the hemolytic agent and just before the animal was sacrificed The spleen and the liver were removed at necropsy and fixed in 10% buffered formalin Paraffin sections 5 μm in thickness were stained with hematoxylin and

[illegible]. An erythrophagocytic count was done on each slide. That is, the number of [illegible] cells in [illegible] high power fields (HPF) were counted and the average per HPF was calculated. The average count at each interval in the spleen and the liver were counted separately and their total count was obtained. 10 control animals were sacrificed to study the normal values.

Results

Copper Sulfate (fig. 1a)

Serum bilirubin. The highest level was observed 24 h after administration of copper sulfate. It was 3.1 mg%. The levels were within normal limits thereafter, that is, 1.5 mg% on the 2nd day, 2.2 mg% on the 3rd day, 2 mg% on the 5th day and 1.9 mg% on the 7th day.

Reticulocyte count. There was hardly any reticulocyte response on the first 3 days, the counts being 0.8% after 24 h, 1.6% after 48 h and 5.9% after 3 days. There was a sudden rise to 32.6% on the 5th day. The count fell rapidly to 4.6% on the 7th day.

Hemoglobin. The normal hemoglobin level is 12.5 g%. After copper sulfate this level was maintained on the first day. The hemoglobin fell first to 9.6 g% on the 2nd day and then to 7.4 g% on the 3rd day. The levels then rose to 10.1 g% on the 5th day and to 10.2 g% on the 7th day.

Erythrophagocytic count (EPC). The total EPC was 31 HPF on the 1st day. There was a gradual fall to 25 HPF after 48 h, 11 HPF after 72 h, 9 HPF after 5 days. No activity was seen on the 7th day. The erythrophagocytic activity was mostly splenic, the liver showing very mild activity on the first 2 days.

[illegible] (fig. 1b)

Serum bilirubin. This was above normal throughout the period of study. The level was 2.91 mg% after 24 h and it rose further to 3.44 mg% after 48 h and 4.00 mg% after 72 h. On the 5th day the level was the same as on the third day and it fell to 3.31 mg% on the 7th day.

Reticulocyte count. It showed a [illegible] as early as 24 h (15%). It then increased to 18% after 48 h, 25.1, 27.5 and 36.0% on the 3rd, 5th and 7th days respectively.

Hemoglobin. The level [illegible] from 12.5 g% to 7 g% [illegible] 24 h. The level was [illegible] g% on the 2nd day [illegible] by a further fall to [illegible] g% on the 3rd day. The level [illegible] mg% on the 5th day and [illegible] [illegible] g% on the 7th day.

Erythrophagocytic count. The total EPC was 21 HPF after 24 h. The

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Splenic Feedback in Red Cell Regeneration

BHANU IYENGAR and KAMLA CHANDRA

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Abstract Equivalent hemolysis was produced in adult albino rats by intracardiac injection of copper sulfate phenylhydrazine and antiserum Reticulocyte response and hemoglobin formation were studied in relation to splenic erythrophagocytic activity, hepatic erythrophago cytosis and evidence of intravascular hemolysis Splen feedback produced an early, rapid reticulocyte response in the hemoglobin levels whereas hepatic erythrop breakdown produced a slower response

The spleen is an important destruction sit
cells It is likely that the red cell levels are
anisms from this organ With this in vie
using different hemolytic agents

Materials an

Adult albino rats of either sex we
ments The doses of three different hen
proximately 3 mg% of serum bilirub
fate produced this degree of hemol
Grade 5 000 µg 200 g body weight)
and anti rat erythrocyte serum ra
weight) The animals were sacrifice
3 5 and 7 days

The serum bilirubin levels re
termined before injecting the he
ficed The spleen and the liver
formalin Paraffin sections 5

Antiserum (fig. 1c)

Serum bilirubin. This was high after 24 h (3.15 mg%) and on the 5th day when the level was 3.21 mg%. The level was 2.74 mg% on the 2nd day, 3.07 mg% on the 3rd day, and 3.19 mg% on the 7th day. Thus hem[illegible] was sustained at a high level with minor fluctuations.

Reticulocyte count. It rose quickly on the 1st and 2nd day, the values being 6.[illegible]% after 24 h and 15.[illegible]% after 48 h. This was followed by a fall to 10% on the 3rd day. There was a rise on the 5th and 7th days to 20.7 and 40.9%.

Hemoglobin. This fell steeply on the 1st day to 5.9 g%. The level rose to 7.5 g% on the 2nd and 3rd day, 8.5 g% on the 5th day and 10.9 g% on the 7th day.

Erythrophagocytosis. After 24 h the EPC was 24 HPF. There was a rise to 31 HPF on the 2nd day. The count fell on the 3rd day to 12 HPF. After a secondary rise to 17 HPF after 5 days the count was 10 HPF on the 7th day. The rate of liver EPC was 7 HPF after 24 h. It rose to 12 HPF after 48 h. After 72 h the level fell to 6 HPF. The EPC was 9 HPF on the 5th day and 6 HPF on the 7th day. The splenic EPC followed a pattern similar to but initially much higher than that of the liver, the counts being 17 HPF after 24 h, 19 HPF after 48 h, [illegible] HPF after 72 h, 8 HPF after 5 days and 4 HPF after 7 days. Here the 1st splenic peak is higher than that of the liver, but the 2nd peak is similar to the hepatic curve.

Controls. The normal serum bilirubin level was 1.75 mg% and the hemoglobin 12.5 g%. The EPC and reticulocyte count was nil.

Discussion

As the RE cells of the spleen take a major part in the breakdown and the processing of effete and abnormal red cells, it is likely that the red cell [illegible] are maintained by feedback mechanisms from this organ. A previous study showed that in intravascular hemolysis the red cell breakdown products are not processed by the spleen [1]. Thus in intravascular hemolysis the feedback for red cell regeneration is not from the spleen. It is known that intravascular red cell breakdown products are an important feedback [2].

It is evident from the results above that, although the initial [illegible] produced after 24 h [illegible] values in all three agents [illegible]

lysis and regeneration vary markedly [3] Copper sulfate produces a predominantly extravascular hemolysis which is mainly splenic The hemolysis is short-lived, showing a peak lasting for the first 48 h As no red cell breakdown products are present in the circulation with this agent, the feedback depends on the erythrophagocytosis by the splenic RE cells

The reticulocyte response is seen after the 3rd day, the peak being on the 5th day The rise is rapid and abrupt and wanes away as quickly The peak level of reticulocytes is equal to that of phenylhydrazine and antiserum, although the hemolysis is low and short-lived The peak reticulocyte levels follow the release of copper into the splenic RE cells [4] Copper is known to stimulate the incorporation of iron into hemoglobin [5] and promotes a rapid proliferation of the RE cells with myeloid metaplasia [6]

There is no active reticulocyte formation up to the 3rd day, whereas the reticulocyte levels start rising immediately with the other two agents The difference is due to the fact that the hemoglobin levels are maintained at a normal level 24 h after copper due to the shock [7] Thus, in spite of a rapid extravascular hemolysis, the feedbacks from the splenic RE cells are only effective when the hemoglobin levels fall

Phenylhydrazine also produces a predominantly extravascular hemolysis which is mainly in the spleen Hepatic erythrophagocytosis is also prominent in the later phases This agent gives rise to a progressive hemolytic curve, so that a high rate of hemolysis is maintained throughout The peak levels are much higher than that of copper sulphate Here again intravascular breakdown products are negligible There is a rapid hemoglobin fall within the first 24 h, and it continues to fall to a lower level till the 3rd day The splenic EPC is high during the first 24 h and maintains a lower plateau on the 3rd and 5th day The liver EPC rises during the later period

The regeneration is in two phases, an early rapid rise and a later slower rise to a peak on the 7th day The early rapid phase coincides with the splenic erythrophagocytosis and starts within 24 h as hemoglobin levels fall rapidly The later slower rise coincides with the hepatic EPC The splenic EPC stimulates an early and rapid feedback for red cell regeneration The peak regeneration is reached on the 7th day, in spite of the rapid fall of hemoglobin an early rapid rise of reticulocytes, and a prolonged hemolysis Previous work indicates that chronic hemolysis plays a part in stimulating rapid regeneration [8]

Antiserum produces both intravascular and extravascular hemolysis,

the main component being intravascular. The early phase is extravascular and dependant mainly on the spleen. This agent also produces a high sustained hemolysis maintained throughout the 7 days. Here the early feedbacks depend on splenic EPC and later the intravascular red cell breakdown products. The earliest part of the reticulocyte curve coincides with that of phenylhydrazine – that is, with the early phase of splenic EPC. There is a rapid and steep fall in hemoglobin levels within 24 h. Later, when the intravascular hemolysis is high and the EPC low, the reticulocyte rise is low following an initial fall on the 3rd day. This fall on the 3rd day occurs in spite of the sustained hemolysis which is intravascular. This indicates that the feedback from the splenic RE cells is strong and a fall in the EPC withdraws this mechanism with a fall in the reticulocyte count. The slower rise during the intravascular phase depends on the red cell breakdown products.

Hence it is seen that the splenic RE cells effect a quick response when accompanied by a decrease in hemoglobin. The red cell breakdown products and the Kupffer cells of the liver, on the other hand, stimulate a slower response. Copper, in addition, stimulates a rapid incorporation of iron into hemoglobin and a proliferation of the RE cells.

Acknowledgements. We are very thankful to the Director-Principal of the Maulana Azad Medical College for giving us permission to publish this work. Our thanks are due to Mr. R. C. [illegible] for the [illegible].

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Phenylhydrazine also produces a predominantly extravascular hemolysis which is mainly in the spleen. Hepatic erythrophagocytosis is also prominent in the later phases. This agent gives rise to a progressive hemolytic curve, so that a high rate of hemolysis is maintained throughout. The peak levels are much higher than that of copper sulphate. Here again intravascular breakdown products are negligible. There is a rapid hemoglobin fall within the first 24 h, and it continues to fall to a lower level till the 3rd day. The splenic EPC is high during the first 24 h and maintains a lower plateau on the 3rd and 5th day. The liver EPC rises during the later period.

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Acta Haemat. 51: 297–301 (1974)

Pathogenesis of Anemia Associated with *Mycoplasma Pneumoniae*

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Departments of Medicine and Pathology, Harbor General Hospital, Torrance; Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif.

Abstract. A 40-year-old female infected with *Mycoplasma pneumoniae* developed severe pneumonia with hypoxia and anemia. Both conditions responded promptly to erythromycin. The patient's serum contained cold agglutinins at a high titer for 10 days. The agglutinins were polyclonal cold agglutinins of [illegible] type and predominantly [illegible]. During the acute phase of illness the patient was anergic to tuberculin. It is suggested that in addition to hemolysis, bone marrow suppression played an important role in the pathogenesis of the anemia in our patient.

Key Words: Cold agglutinins · Erythromycin · Hemolytic anemia · *Mycoplasma pneumoniae*

Recent literature, with a few exceptions [1, 2], lacks reports of atypical pneumonia complicated by hemolytic anemia, although such association was relatively common in earlier studies [3, 4]. This study describes a patient with severe pneumonia and anemia who had cultural and serological evidence of *Mycoplasma pneumoniae* infection and who responded promptly to erythromycin.

Case Report

A 40-year-old [illegible] female was admitted to Harbor General Hospital because of weakness, chest pain [illegible] and shortness of breath. [illegible]

[illegible]

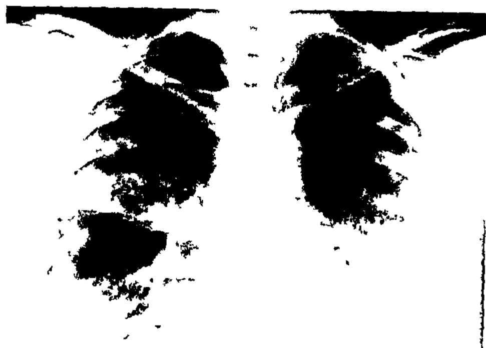


Fig 1 Posteroanterior chest X ray (First day of hospitalization)

ia associated with weight loss. Thirty years ago she worked in a TB sanatorium and had a positive PPD test but her chest X ray films have been negative except for a few calcified nodes. Physical examination: obese white female in respiratory distress. The temperature was 101 °F (orally), pulse 96, respirations 20. Blood pressure 130/80 mm Hg. There were coarse rales at the left base but no dullness on percussion. Hematocrit 37.3%, hemoglobin 12.8 g/100 *ml*. White cells 21 200 *mm*^{3} with 68% segmented and 7% band neutrophils, 12% lymphocytes, 10% monocytes, 2% eosinophils and 1% basophils. Routine urinalysis and urine culture were negative. Gram stain of sputum: many neutrophils but few bacteria; cultures grew normal flora. Arterial blood gasses while the patient was receiving oxygen (2 l/min): Po_2 72 *mm* Hg, Pco_2 28 *mm* Hg, *p*H 7.48. Total bilirubin 1.6 mg/100 ml, 0.1 mg/100 ml direct. Skin tests with intermediate and second strength *PPD*, *histoplasmin and coccidiodin* antigens were negative. Chest X ray: diffuse infiltrates involving the right upper lobe, the lingula and superior portions of the left lower lobe (fig 1). The patient was treated initially with penicillin, isoniazid, ethambutol and streptomycin but continued to be febrile up to 105 °F. Two days after admission the leukocyte count rose to 45 900 mm^3. Three days after admission her hematocrit dropped to 25.7%. Platelet number appeared normal. Corrected reticulocyte count was 3.7%, total bilirubin 1.8 mg/100 ml, serum haptoglobin 170 mg/100 ml, serum *iron 60* μg

[illegible] of 225 mg/100 mL. Urinary hemosiderin was positive [illegible] and there was no hemo[illegible]. Four days after admission, bone marrow showed an increased myeloid [illegible]. The peripheral blood showed [illegible]. Two days after admission, a [illegible] was found positive; the patient was started on erythromycin 2 g daily. [illegible] later the patient deteriorated, and [illegible] serum bilirubin fell to 0.8 mg/100 mL and hemo[illegible] and [illegible]. A [illegible] peak was found 3 days after the erythromycin was started. On the 11th day of hospitalization the patient had [illegible]. FPD [illegible] days after admission, the [illegible] Coombs test was positive. Two months after discharge the patient complained of dyspnea. Her hematocrit was 41% and the [illegible] were [illegible]. [illegible] and [illegible] were [illegible] normal. Chest X-rays revealed a persistent small [illegible] in the left upper lobe.

M. pneumoniae [illegible] was isolated [illegible] and [illegible] from the [illegible] specimen taken on the fourth day of hospitalization. The organism was identified as *M. pneumoniae* on the basis of [illegible]. [illegible] *M. pneumoniae* [illegible] and the [illegible] on the 17th day [illegible] of 2,048.

Cold agglutinin [illegible] was found on the [illegible] day of hospitalization. The titer decreased to [illegible] on the [illegible] day [illegible] on the [illegible] and it became negative on the [illegible] day. The cold agglutinin [illegible] from the first specimen [illegible] found by [illegible] at 2 [illegible] of agglutinin [illegible] cold agglutinin activity was present in the [illegible] peak on [illegible] corresponding to IgM. Light chains of the agglutinin were predominantly [illegible]. The cold agglutinin was [illegible] of [illegible] when examined by [illegible]. The [illegible] cold agglutinin [illegible]. The [illegible].

Discussion

The [illegible] of *Mycoplasma pneumoniae* [illegible] was [illegible] [illegible] and [illegible] [illegible] FPD [illegible] *M. pneumoniae* [illegible] [illegible] of *M.* [illegible]



Fig. 1 Posteroanterior chest X ray (First day of hospitalization)

ia associated with weight loss Thirty years ago she worked in a TB sanatorium and had a positive PPD test but her chest X ray films have been negative except for a few calcified nodes Physical examination obese white female in respiratory distress The temperature was 101 °F (orally) pulse 96 respirations 20 Blood pressure 130 80 mm Hg There were coarse rales at the left base but no dullness on percussion Hematocrit 37 3% hemoglobin 12 8 g 100 ml White cells 21 200 mm³ with 68% segmented and 7% band neutrophils, 12% lymphocytes 10% monocytes, 2% *eosinophils, and 1% basophils Routine urinalysis and urine culture were negative* Gram stain of sputum many neutrophils but few bacteria cultures grew normal flora Arterial blood gasses while the patient was receiving oxygen (2 l min) P_{O_2} 72 mm Hg P_{CO_2} 28 mm Hg pH 7 48 Total bilirubin 1 6 mg 100 ml 0 1 mg 100 ml direct Skin tests with intermediate and second strength PPD histoplasmin and coccidiodin antigens were negative Chest X ray diffuse infiltrates involving the right upper lobe the lingula and superior portions of the left lower lobe (fig 1) The patient was treated initially with penicillin isoniazid ethambutol and streptomycin but continued to be febrile up to 105 F Two days after admission the leukocyte count rose to 45 900 mm³ Three days after admission her hematocrit dropped to 25 7% Platelet number appeared normal Corrected reticulocyte count was 3 7% total bilirubin 1 8 mg 100 ml serum haptoglobin 170 mg/100 ml serum iron 60 μg

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[4] and, except for a few isolated cases [1, 2], pneumonia due to *M pneumoniae* has not been commonly accompanied by manifest hemolysis [9]

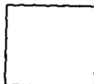
Elevated cold agglutinins (1 640 to 1 16,000) were more common among severe cases of pneumonia and among cases complicated by hemolytic anemia [3], yet many patients with high cold agglutinin titer did not develop anemia Cold agglutinins are believed to be a result of immunization with antigens cross-reacting with red cells and found on certain microorganisms such as *M pneumoniae* [10]

Following the administration of erythromycin, patient's serum bilirubin became normal in 3 days and hematocrit rose in 5 days The prompt effect of erythromycin, a highly effective antibiotic against *M pneumoniae*, on both the pulmonary involvement and the anemia thus suggests a direct role for *M pneumoniae* in both conditions In addition to hemolysis due to cold agglutinins, bone marrow depression may have been important for the following reasons (1) cold agglutinins were still elevated while the hematocrit was returning to the normal level, (2) reticulocytosis actually increased following the erythromycin therapy which was accompanied by a rising hematocrit, (3) the bone marrow manifested lack of erythroid hyperplasia in the face of a significant anemia The temporary suppression of delayed hypersensitivity to tuberculin may reflect inhibitory effect of mycoplasma on thymus-derived lymphocytes

This report adds one case to the 16 cases of atypical pneumonia with immunochemical studies of the associated cold agglutinin reported in the literature and reviewed previously [6] Transient postinfectious cold agglutinins are either polyclonal or restricted polyclonal when studied by immunoelectrophoresis and light-chain typing The cold agglutinins in this patient were polyclonal and lacking the cold agglutinin idiotype determinant, reflecting stimulation of multiple clones of cells but not those which are derepressed in patients with chronic idiopathic cold agglutinin disease The anti-I specificity of the cold agglutinin was demonstrated by a strong reaction with I-positive red cells and a negative reaction with cord and I-negative adult red cells, and by complete neutralization of agglutination using the I substance in boiled human milk The H substance in boiled saliva neutralized the agglutinin much less than the I substance The agglutinin appears therefore to be a cold acting anti-I with little or no H specificity

Acknowledgements The authors thank Dr K TANAKA Harbor General Hospital for performing assays of intracellular red cell enzymes

Acta Haemat 51 302-309 (1974)



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Abstract A case of
myeloma protein is de
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In recent years,
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Fig 1 Skull punched out osteolytic lesions

phology which in some areas looked clearly punched out in others they showed blurred rims In some places the cortex of the bone looked dented for an endosseous process of medullary origin In these areas the external aspects of the cortex did not appear insufflated and the external aspects of the cortex did not appear insufflated and the external surface of the bone was preserved Tibiae and fibulae showed uniform and diffuse osteoporosis Plasmocytosis was found in the *bone marrow* (fig 2) *Electrophoresis of the serum* showed a clear diminution of γ globulins

The patient was later admitted to the Institute of Patologia Medica R', University of Genoa The laboratory findings are summarized in table I A search for cancer metastases that might cause the described osteolytic lesions was negative X ray examinations of the thorax gastrointestinal tract and urinary system were negative Thyroidal ^{131}I uptake 42% after 2 h 61% after 8 h 59.5% after 24 h A regular pattern was seen in the thyroid scanning The renal hippuran scanning showed a normal distribution Gynecological examination was negative

The diagnosis of plasmocytoma was therefore sustained and further investiga

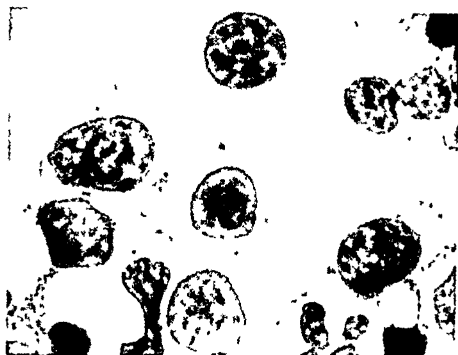


Fig 2 Bone marrow several plasma cells may be observed Their cytoplasm is large and vacuolated The nucleus is in some instances finely granulated and displays nucleoli × 1,200

tions in this field were performed A new biopsy of the bone marrow showed normal cells However among them were several plasma cells (5-6 per microscopic field at × 1 000) with nuclei at times exhibiting fine granules nucleoli and a large cytoplasm stained in reddish blue (flaming)

The bone marrow was collected in tubes containing dry EDTA marrow crumbs were then separated and washed in phosphate buffer at pH 7 2 and BSA three times Slides were then prepared and incubated with antisera to immunoglobulin fractions (A G D E,M) and light chains all of them conjugated with FITC In no test was fluorescent material seen in the cytoplasm of cells Serum electrophoresis in repeated controls showed low levels of γ globulins Immunoelectrophoresis against total antihuman serum and against anti light chain antisera allowed identification of a small precipitation line corresponding to IgG However it had normal morphology Precipitation lines corresponding to other Ig fractions were absent (fig 3)

The agarose plate determination of serum immunoglobulins performed several times constantly showed 400-600 mg 100 ml IgG Other fractions of immunoglobulins were consistently absent The search for urinary paraproteins performed by immunoelectrophoresis of urine concentrates on Sartorius membrane against specific antisera to Bence Jones proteins was consistently negative

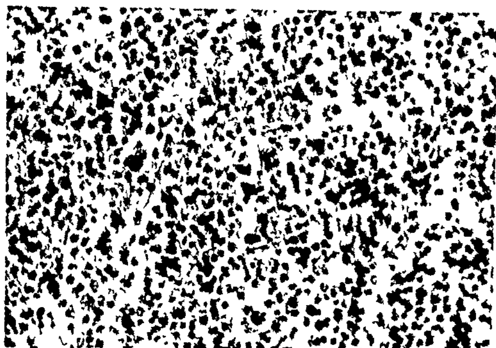


Fig. 4. Histological section of a rib fragment: cluster of lymphoid cells and plasma cells. × 140.

cells involved in this kind of neoplasia is genetically dependent, seems untenable, since both corroborating and disproving data are lacking.

However, our findings cannot exclude the hypothesis mentioned above concerning production by neoplastic cells of a protein different from the known immunoproteins. But the results of serum electrophoresis exclude the presence of relevant amounts of any homogeneous protein. Diminution of an electrophoretic γ-globulin fraction and IgG, and the absence of other immunoglobulins are also remarkable. A hypogammaglobulinemia existing prior to the disease can be excluded.

In attempting to interpret these data, the hypothesis may be advanced that neoplastic cell proliferation induces an inhibition of normal plasma cell clones, consequently impairing production of immunoglobulins and reducing their amount in the serum. A similar behavior is typical for paraproteinemic forms of plasmacytoma. These plasmacytoma patients exhibit large amounts of serum M globulins, but show a diminution of normal immunoglobulins, causing an immune 'paralysis' leading to bacterial infections [3, 5]. It must also be stressed that in the patient studied, even

in the presence of a typical skeletal and marrow pathology, visceral lesions were absent. Functional alteration of various organs (kidney, lungs, nervous system) and impairment of blood clotting could not be detected and no visceral lesions were found at the necropsy. These findings might be ascribed to the absence of a paraproteinemia.

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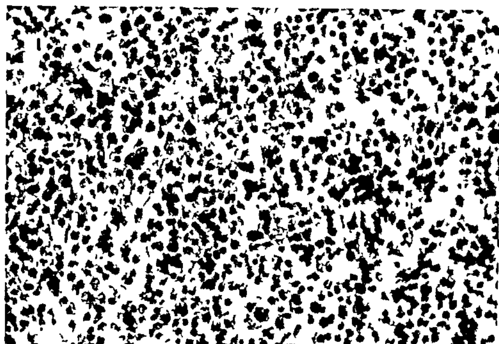


Fig. 4 Histological section of a rib fragment cluster of lymphoid cells and plasma cells × 140

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Acta Haemat 51 310–314 (1974)

Glucose-6-Phosphate Dehydrogenase Jackson

A New Variant Associated with Hemolytic Anemia[1]

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Division of Hematology University of Mississippi School of Medicine Hematology Research Laboratory Jackson VA Center Jackson Mississippi and the Division of Medicine City of Hope National Medical Center Duarte Calif

Abstract A 66-year old man presented with moderately severe hemolytic anemia Hematologic and biochemical evaluation proved him to have a hitherto undescribed variant of erythrocyte glucose 6-phosphate dehydrogenase Glucose 6-phosphate dehydrogenase Jackson is characterized chiefly by chronic hemolysis in the absence of oxidant drugs or infection markedly reduced enzyme activity and variations in substrate affinity In addition the patient had leukopenia and thrombocytopenia possibly resulting from stem cell competition due to the marked demand for erythropoiesis

Key Words
Erythrocyte enzymes
G 6-PD Jackson
Hemolytic anemia

Erythrocyte glucose 6 phosphate dehydrogenase (G 6 PD) deficiency as a cause of drug induced hemolytic anemia was first described in 1956 [6] Subsequent studies have shown that this enzyme displays marked heterogeneity as regards its activity, substrate affinity, stability, pH optima and electrophoretic mobility This communication describes a clearly distinct new variant designated *G 6-PD Jackson* which is associated with moderately severe chronic hemolytic anemia

Case Report

CD a 66-year-old white man was admitted to the University of Mississippi Hospital in May of 1972 for evaluation of anemia He was in good health until 7 years prior to admission when during the course of hospitalization for treatment of lum

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the partially purified enzyme was 32 6 μM, the K_m NADP (measured spectrophotometrically) was 6 15 μM, the K_i for NADPH at pH 7 1 and 37 °C was 80 mM (normal 20–30 μM), and the pH optimum curve was normal Thermal stability of the dialyzed enzyme was measured at 46 °C. Its residual activity was 86, 73 and 78% respectively, at 20 40 and 60 min (control values 73, 62 and 61%) 2-Deoxy-glucose-6-phosphate utilization was 2 8% of G-6 P utilization, deamino-NADP utilization was 66 7% of NADP utilization The electrophoretic mobility of the enzyme was 102 4% of B in the EDTA-borate tris system, 100% of normal in the tris HCl system and 104 5% of B in the phosphate system [14]

Red cell ^{51}Cr $t^1/_2$ was shortened to 7 days The $t^1/_2$ of injected ^{59}Fe was rapid (23 min) plasma Fe-turnover was increased to 4 35 mg Fe/100 ml blood/24 h, erythron ^{59}Fe-turnover was 3 96 mg Fe/100 ml blood/24 h and red cell ^{59}Fe-utilization was diminished to 18 7% with an early peak at 7 days

Fifty family members were investigated (21 males and 29 females) All studied had normal G-6-PD activity of their erythrocytes as well as normal values for hemoglobin concentration, hematocrit and reticulocyte count

Comment

There are over 100 reported variants of G 6-PD [2 4 7, 15–17], characterized by enzyme activity, substrate affinity, heat stability, pH optima and electrophoretic mobility G-6-PD Jackson is associated with moderately severe chronic hemolytic anemia markedly reduced enzyme activity, and variations in substrate affinity

The patient s reticulocyte count was 40–50% during 6 months of observation while measurements of red cell mass and hematocrit remained constant The myeloid erythroid ratio (M/E) was 1 40, and increase of approximately 60 fold when compared to a more usual M E of 3 2 The erythroid production index calculated from the reticulocyte count, hemoglobin concentration and reticulocyte life span was 16 times normal [12] Although care was taken to exclude counting erythrocytes with precipitated hemoglobin as reticulocytes, in cells with a sparse reticulum this was difficult The reticulocyte count and production index may therefore, be somewhat lower than our estimates Plasma iron turnover was increased and total erythron iron turnover was 3 96 mg Fe 100 ml of blood 24 h [8] Red cell utilization peaked early and was diminished These findings

are consistant with hemolytic disease, although it may be difficult to separate the effects of hemolysis and ineffective erythropoiesis on ferrokinetics. The high reticulocyte count, increased production index, and only moderately elevated LDH suggest that significant ineffective erythropoiesis was not present.

Additional instances of enzyme deficiency were not detected during family studies; however, assays of enzyme activity alone do not always detect female heterozygotes. Our failure to find additional affected individuals most likely resulted from chance, although a mutation originating in the patient cannot be excluded.

Repeated measurements of leukocyte and platelet counts were consistently at or below the lower limits of normal. The activity of G-6-PD has been noted to be decreased in both leukocytes [13] and platelets [9] of some individuals with G-6-PD deficiency, although this has not been associated with cytopenias. The normal size of the circulating platelets and the lack of immature granulocytes in the blood, associated with the bone marrow findings, suggest that the destruction rate of these elements was not accelerated, although survival studies were not performed. A demand for marked augmentation of erythropoiesis has been shown to cause increased input into the erythroid committed stem cell pool from the pool of pluripotent stem cells [11]. This is accompanied by diminished granulopoiesis, presumably on the basis of stem cell competition. The marked erythroid activity of the marrow in our patient is consistant with such a mechanism underlying the mild leukopenia and thrombocytopenia we observed.

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Hemoglobin S-G Georgia Disease: A Case Report

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Abstract. A 7-year-old Black American boy with severe [illegible] anemia, mild hepatomegaly and reticulocytosis [illegible] had a persistent and rather severe crisis which was in part responsible for the anemia. Electrophoretic, chromatographic and chemical analyses indicate the simultaneous occurrence of Hb S and Hb G Georgia or α_2(95 Pro→Leu)β_2. The father is a Hb S heterozygote and the mother a Hb G heterozygote. Quantitative analyses of the patient's hemoglobin show the presence of about 15% α^G containing hemoglobin and about 38% β^S containing hemoglobins which is about the same as is observed in a Hb S trait individual.

Key Words
Hb G Georgia
Hb S-G Georgia
Hemoglobin interactions
Hemoglobinopathies
Sickle cell anemia

The simultaneous occurrence of sickle cell hemoglobin (Hb S) and other β chain variants such as Hb C, Hb O Arab, Hb D Los Angeles [1], or α chain variants such as Hb G Philadelphia [2] has been observed repeatedly. Clinical symptoms attributable to the presence of the two variants are often uncommon, particularly in infancy. However, the SC, SD Los Angeles, and the SO Arab diseases can be severe from early childhood, although in the majority of cases the clinical course is mild [1]. We recently observed a child who has the Hb S-G Georgia condition. Hb G Georgia is an α chain variant with an amino acid substitution (95 Pro→Leu) at the $\alpha_1\beta_2$ contact interface which results in distinct changes in functional properties [3, 4].

Methods

Hematological analyses were made [illegible]. Red cell [illegible] were made by washing [illegible] and lysing the [illegible]

volume of distilled water and 0.2 vol of carbon tetrachloride. Stroma and other debris were removed by centrifugation at 10 000 g and at 4 C for 20 min.

Starch gel electrophoresis used the method of EFREMOV *et al.* [6]. Analytical and preparative DEAE Sephadex and CM-cellulose chromatography followed previously described procedures [7–9]. Identification of the abnormal hemoglobin fractions was made by techniques described in earlier communications [3, 4]. The stability of hemoglobin in hemolysate against heating was determined by the method outlined previously [10].

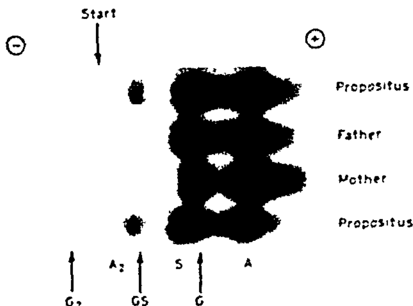
Case Report

J. L., a 7 year-old black male, was first seen at the age of about 2 years for spontaneous nosebleeds. Anemia was evident then and has persisted. The nosebleeds occur once or twice a week and appear to be brought on by heat or cold. In March 1967 he was admitted with a PCV value of 14%, at which time he received one unit of packed red cells. Serum iron and TIBC at time of admission were 33.2 and 513 µg%, respectively. The anemia improved on treatment with ferrous sulfate and the patient has been on this regimen ever since the diagnosis of anemia was first made. However, he has never been able to obtain a PCV value higher than 33%, with reticulocyte counts ranging between 3.2 and 12.5%. A mild hepatomegaly was present. Hematological data are indeed most variable: at his visit in the fall of 1972, the values were: PCV 29%, Hb 9.8 g%, Retics 8.2%, whereas in the spring of 1973 the data were: PCV 17%, Hb 5.5 g%, RBC 3.04 × 10⁶/mm³, WBC 4 200, MCV 55 µm³, MCH 18 pg, MCHC 30%. One month later, after ferrous sulfate treatment, the values were: PCV 33%, Hb 10.9 g%, Retics 3.2%. These observations made it likely that the anemia was to a major extent caused by iron deficiency resulting from the rather severe and repeated nosebleeds. Despite extensive evaluation, the cause of this epistaxis remained unclear. Heinz body formation was not remarkable although a slightly increased susceptibility in the presence of an oxidant drug was noted. The patient did not have glucose-6-phosphate-dehydrogenase deficiency.

Family studies. The propositus is one of 6 children. Lack of parental cooperation has made it impossible to study the other 5 siblings. The father has a sickle cell trait and the mother is heterozygous for Hb G*a* Georgia. The mother maintains a normal PCV value averaging 44%, with a slight reticulocytosis of about 2.5% and a normal RBC morphology except for a few target cells and a slight polychromatophilia.

Hemoglobin analyses. Electrophoretic examination of hemoglobin from the propositus showed the presence of five components (fig. 1). One component had a mobility similar to that of Hb A, a second resembled Hb S, and a third was Hb A_2, whereas a fourth and a fifth component moved slightly faster and slightly slower than Hb A_2, respectively. The electrophoretic pattern of the hemoglobin of the father is typical of that of a sickle cell trait individual. The hemoglobin of the mother showed four distinct bands: one in the position of Hb A, a second in the position of Hb A_2, a third in the approximate position of Hb S, and a fourth component which moved slightly slower than Hb A_2. Heat denaturation curves of hemoglobin from the propositus, his father, and a normal control were made at 50, 62, and 70 C. No differences were observed between the three hemolysates, thus suggesting a normal heat stability of the Hb G and/or the Hb GS components.

Quantitation of the hemoglobin fractions was made on columns of DEAE Sephadex


Start
Propositus
Father
Mother
Propositus
A2
S
A
G2
GS
G

[illegible]

[illegible]

[illegible]

[illegible]

Table I The percentages of the various hemoglobin components in the propositus and his parents

Hb component	Propositus[1]	Father[2]	Mother[1]
G_2	0.7		0.8
A_2	2.0	2.9	2.5
A	57.3	61.1	82.2
S	26.0	36.0	
G	11.4		14.5
GS	2.6		
Hb with α^G	14.7	0	15.3
Hb with β^S	28.6	36.0	0

[1] By CM-cellulose chromatography. The minor hemoglobins A_2 and G_2 of the propositus do not separate from Hb S and Hb GS, respectively. The quantities of Hb A_2 and Hb G_2 were determined by DEAE-Sephadex chromatography and those of Hb S and Hb GS corrected.

[2] By DEAE Sephadex chromatography.

Comments

When an abnormal hemoglobin in either α or β chain is present together with Hb S, an interaction may occur which can influence the effects of the sickling process. In Hb Memphis, for instance, position 23 of the α-chain is occupied by a glutaminyl residue in lieu of a glutamyl residue [11]; this substitution acts to reduce sickling in erythrocytes containing Hb S. When β-chain variants are present, an interaction can also be observed. Hb Richmond [6] and Hb C Harlem [12] appear to reduce the *in vivo* sickling phenomenon, whereas Hb O Arab and Hb D Los Angeles (for references, see [1]) can interact with Hb S to produce a moderate to severe anemia.

Evaluation of the possible interaction of Hb *Ga Georgia* with Hb S *in* the propositus and thus of the effect on the clinical condition of this young patient is almost impossible to make mainly because of the persisting and severe epistaxis, the marginal care at home and lack of parental cooperation. The child is for all general purposes a sickle cell trait individual because the percentage of Hb S is the same as that of his father. Also, the presence of Hb S does not appear to influence the synthesis of the α^G-chain. One is inclined to speculate about a possible effect of the interaction between the

β^S-chain and the α^G-chain. However, it is indeed difficult to assume that the presence of some 15% α^G-chain in a Hb β^S heterozygote can cause a clinical condition resembling a fairly severe form of sickle cell disease unless the interaction between these chains (in the form of the GS component) causes a rapid and continued hemolysis. No such effect has become apparent. It is indeed regrettable that lack of material prevented us from collecting data on the effect of the $\alpha^{95 Pro \to Leu}$ substitution on the gelling properties of Hb S. Hopefully such data can be made available when the condition of the patient improves due to intensified medical care.

Acknowledgement. This research was in part supported by US Public Health Service Research Grants HL-05168 and HL-15158.

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Fibrinogen/Fibrin Degradation Products and Factor XIII

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Abstract. The effect of fibrinogen/fibrin degradation products (FDP) on the stabilisation of fibrin was tested in vitro by the clot solubility assay and by the transamidase assay of 14C glycine-ethylester incorporation into casein. No significant interference with the action of factor XIII by FDP was demonstrated. FDP prepared in vitro could act as a substrate for factor XIII although compared with casein FDP are a poor substrate. Patients with high titres of FDP in the serum form normal insoluble clot. Defective fibrin stabilisation does not appear to be important in the bleeding disorders seen in disseminated intravascular coagulation, even though both high titres of FDP and low levels of factor XIII are found.

Key Words
Bleeding disorders
Disseminated intravascular coagulation
Factor XIII
Fibrin degradation products
Fibrin stabilisation

In normal plasma fibrin polymers formed following the limited proteolysis of fibrinogen by thrombin are stabilised by the formation of covalent bonds between adjacent polymers, under the influence of the enzyme fibrin stabilising factor, now known as factor XIII [17, 20, 22, 24, 32]. This appears to be an essential step for normal haemostasis since inherited deficiency of factor XIII, a condition in which fibrin stabilisation does not occur, is characterised by a severe and often fatal bleeding diathesis [3, 4, 6, 8, 11, 13, 21, 33].

Clot formed in the presence of fibrinogen/fibrin degradation products (FDP) is unusually loose and friable and has a grossly abnormal structure as shown by electron microscopy [1]. The abnormality is caused by inhibition of normal polymerisation because FDP compete with fibrin monomers for the polymerisation sites and are incorporated into the clot structure [14, 15, 16]. Although it might be expected that clot which is [illegible]

modified by incorporated FDP would prove to be an unsuitable substrate for factor XIII, the effect of FDP on the process of fibrin stabilisation has not, to our knowledge, been investigated

We have studied the action of FDP on fibrin stabilisation *in vitro*, by determining the effect of FDP, present during clotting, on the solubility of normal clot and the effect of FDP on the action of factor XIII as measured by a sensitive assay, and we have assessed the possibility of FDP acting as substrate for factor XIII We have also studied fibrin stabilising activity in the plasma of 4 patients with high plasma levels of FDP and an accompanying haemorrhagic diathesis compatible with the diagnosis of disseminated intravascular coagulation (DIC)

Materials and Methods

Thrombin (S Maw Son & Sons Ltd, Barnet, England) 50 U/ampoule Streptokinase, recently outdated Kabikinase (Kabi Pharmaceuticals Ltd London) Rabbit anti human fibrinogen serum (Meath Paines & Byrne Greenford UK) Agar ID agar tablets (Oxoid Ltd London) Trasylol (Aprotinin 25 000 KIU/ml FBA Pharmaceuticals, Haywards Heath, Sussex, UK)

FDP were estimated using the Wellcome FDP Kit (Wellcome Reagents Ltd Beckenham, England) and the Microtiter equipment (Cooke Engineering Co., California, USA) The method is based on the principle of the tanned red cell agglutination inhibition immunoassay of MERKSEY *et al* [27]

Electrophoresis of FDP was carried out in Shandon electrophoresis equipment using 1% agar in 0 05 M veronal buffer at constant voltage (180 V) for 4 h Immunodiffusion of the separated bands was with anti human fibrinogen (Meath) diluted 1 2 with veronal buffer, for 36 h at 4 °C. After exhaustive washing with normal saline, the plates were dried at room temperature and stained with amido black

Preparation of FDP Fibrinogen free of factor XIII was prepared from the plasma of a patient with inherited deficiency of factor XIII (case No 1 LOSOWSKY *et al* [23]), by the method of BIGGS and MACFARLANE [5] The preparation contained 79% of clottable protein and formed clot which dissolved immediately in 1% monochlor acetic acid The factor XIII free fibrinogen was dissolved in Tris buffer (0 05 M pH 7.5), and incubated at room temperature with streptokinase at a final concentration of 25 U/ml Fibrinogen breakdown was followed by observation of the influence of the mixture on the thrombin clotting time of normal plasma as follows 0.2 ml normal plasma was mixed with 0 1 ml Tris buffer (0 05 M pH 7.5) or 0 1 ml of the fibrinogen digest mixture and 0 1 ml of thrombin solution (5 U/ml) added Control clotting times were 8–9 sec under these conditions. Figure 1 shows the time course of the anti thrombin activity of the fibrinogen digest mixture Just before the peak of anti thrombin activity was reached (14 min of incubation) the mixture became unclottable At the peak of anti thrombin activity half the mixture was withdrawn, and plasmin activity inhibited by adding an excess of Trasylol This portion

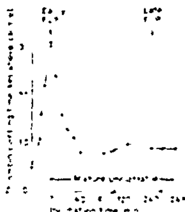


Fig. 1. Development of antithrombin activity in the fibrinogen-streptokinase mixture. At peak antithrombin activity half of the mixture was removed, plasmin activity inhibited by excess Trasylol, and the mixture freeze-dried (early FDP). Incubation was continued until stable antithrombin activity was reached. The mixture was then partly purified and freeze-dried (late FDP).

was freeze-dried for future use and its antithrombin activity remained stable. The remainder of the mixture was allowed to incubate for a further 4 h so that the final products of digestion of fibrinogen could form. These products were partly purified by the method of [illegible] et al. [illegible] and then freeze-dried. The nature of the FDP present was confirmed by immunoelectrophoresis which showed that the first part of the mixture contained mainly large early fragments (probably mainly fragment 'X' which is the major constituent at peak antithrombin activity [illegible]) which as [illegible] have a mobility very similar to fibrinogen [illegible] and the second part, the final faster moving products of digestion (probably mainly fragments 'D' and 'E') [illegible]. The FDP in the first part of the incubation mixture were designated early FDP and those in the second part of the incubation mixture late FDP. Immunoelectrophoresis of sera from the 4 patients under the same conditions showed a pattern [illegible] similar to that of early FDP.

Factor XIII assay. The transamidase activity of factor XIII was measured by the [illegible] glycine ethyl ester casein assay previously described [illegible]. In some experiments [illegible] solutions of early FDP and late FDP were used as substrate instead of casein to see whether factor XIII can incorporate [illegible] glycine ethyl ester into FDP fragments, i.e. if they were a suitable substrate for the enzyme. In other experiments FDP solutions were preincubated with plasma before the assay to see if they had any inhibition of factor XIII. The effect on the assay of Trasylol and streptokinase, some of which might be present in the FDP solutions, was also tested.

Control [illegible] 0.2 ml of [illegible] plasma was diluted with 0.2 ml Tris

Table 1 Four patients with high titres of FDP associated with DIC in whom fibrin stabilisation was studied

Case No	FDP µg/ml	Fibrinogen mg/100 ml	Platelets $\times 10^3$/mm^3	ELT min	RBC fragmentation	Factor XIII normal %	FDP immuno-electr	Diagnosis
1	>320	159	33	–	+++	14	early FDP	carcinomatosis carc. colon microangiopathic haemolytic anaemia
2	>320	114	121	>500	++	7.5	early FDP	multiple venous thromboses septicaemia collagen disease
3	160	177	187	500	–	6.0	early FDP	acute myeloid leukemia
4	160	193	25	415	–	31.0	early FDP	acute myeloid leukaemia
Normal	< 20	170–410	>150 000	150–300	–	112±33(SD)	–	

buffer (0.05 M pH 7.5). This was clotted by the addition of 0.1 ml of freshly prepared thrombin solution (50 U/ml) in 0.025 M calcium chloride. The clot was incubated for at least an hour to allow stabilisation to take place and was then suspended in a freshly prepared solution of 1% monochloroacetic acid (3 ml). An unstable clot (such as is obtained from plasma of a patient deficient in factor XIII) dissolves within minutes under these conditions [2]. A normal clot does not dissolve even after more than 24 h. Control plasmas from a factor XIII deficient patient and a normal subject were included in each batch. When testing the effect of FDP on stabilisation, FDP solutions in Tris buffer (30 mg/ml) (0.1 ml) were added to this system instead of 0.1 ml of Tris buffer.

Euglobulin lysis time. The plasma was treated as recommended by VON KAULLA [16] and the lysis time determined in the Clot Lysis Time Recorder (Medicon UK Ltd).

Fibrinogen. Thrombin clottable fibrinogen was estimated by the method of GRANNIS [12].

Patients. In 4 patients (two with leukaemia, one with carcinomatosis and one with septicaemia) a grossly elevated titre of FDP (160 µg/ml or over) was found (normal: up to 20 µg/ml). The 4 patients all had cutaneous bruising, bleeding from venepuncture sites and epistaxes, and these together with other features (table I) suggest that they had DIC. Blood was collected from these patients in the fasting state between 7 and 7.30 in the morning without venous occlusion. For fibrinogen and euglobulin lysis time (ELT) estimation, blood was collected into citrate (9 ml of blood to 1 ml of 3.8% trisodium citrate). The portion for ELT estimation was immediately dropped into a thermos flask containing ice water and the euglobulin frac

Table II. Effect of FDP on clot solubility in normal and factor XIII deficient plasma

Factor XIII deficient plasma ml	Normal plasma ml	Tris buffer (0.05 M, pH 7.5) ml	Early FDP ml	Late FDP ml	Calcium[1] thrombin ml	1% MCA[2] ml	Clot[3] 0.25 h	1 h	24 h
0.2		0.2			0.1	3	0	0	0
0.2		0.1	0.1		0.1	3	0	0	0
0.2		0.1		0.1	0.1	3	0	0	0
	0.2	0.2			0.1	3	+	+	+
	0.2	0.1	0.1		0.1	3	+	+	+
	0.2	0.1		0.1	0.1	3	+	+	+

FDP solutions were 30 mg/ml in Tris buffer.
[1] 50 U/ml thrombin in 0.025 M calcium chloride.
[2] 1% monochloroacetic acid.
[3] Clot: 0 = dissolved; + = not dissolved.

tion was precipitated within half an hour of collection. For factor XIII estimation blood was collected into 2% Na EDTA (9 ml of blood to 1 ml of Na EDTA). For the estimation of FDP blood was collected into Trasylol (100 U/ml of blood).

Results

The effect of FDP on fibrin stabilisation by normal plasma in vitro is shown in table II. It can be seen that neither early FDP nor late FDP disrupt stabilisation; early FDP and late FDP had no stabilising effect of their own when added to factor XIII deficient plasma.

Preincubation of plasma with early or late FDP solutions does not affect factor XIII activity as measured by [14]C glycine-ethylester incorporation into casein (fig. 2). Addition of Trasylol and streptokinase had no measurable effect (fig. 2).

Early or late FDP can be linked to [14]C glycine-ethylester by factor XIII and thus appear to act as an acceptable substrate for the enzyme in vitro (fig. 3). It can be seen that with the concentrations used, incorporation was 21% of that into casein with early FDP (line C fig. 3) and 7.2% of that into casein with late FDP (line D).

In the 4 patients the highest titres of FDP (> 1:64) [illegible] were most likely to [illegible] of DIC (table II). [illegible] 4 had plasma fibrinogen towards the lower limit of normal. 2 had severe thrombocytopenia and in the [illegible]

ably to the active sites of factor XIII and thus inhibit its action. Our findings, however, give no evidence that any of these mechanisms is of importance. The presence of FDP, even in high concentration, does not significantly disrupt the process of stabilisation. No irreversible binding of FDP to factor XIII could be shown, and FDP did not affect ^{14}C-glycine-ethyl-ester incorporation into casein or interfere with the formation of stable clot *in vitro*. Similarly, in 4 patients with high titres of FDP in their sera no abnormality of fibrin stabilisation was found, even though 3 of the 4 patients had low factor XIII activity as measured by the ^{14}C-glycine-ethyl-ester assay, a finding which is quite characteristic of DIC [28]. It is well recognised that very low levels of factor XIII (of the order of a few percent of average normal) [2, 29, 35] are sufficient for stabilisation to occur, and it appears that the presence of FDP, even in high titre, does not significantly affect the process.

The clot solubility test as used by us, allowing 1 h of incubation after clotting for stabilisation to occur, would only detect major degrees of inhibition. *Modifying the assay by the use of shorter incubation times and/* or subdilution of the test plasma might uncover minor degrees of inhibition by FDP, which would not, however, be of clinical importance. The transamidase action of factor XIII as measured in the ^{14}C-glycine-ethyl-ester-casein assay may not be identical with its action in clot cross-linking [34], although the two phenomena do go hand in hand in clinical situations [28, 36]. Thus, while the demonstration of lack of inhibition by FDP under the conditions of this assay is highly suggestive that fibrin stabilisation *in vivo* is normal, this cannot necessarily be assumed. With these reservations our results indicate that FDP do not interfere with the action of factor XIII to any major extent and thus it is unlikely that defective fibrin stabilisation plays a significant part in the haemostatic defect seen in conditions such as DIC, where high titres of FDP and low *levels of factor XIII may be found*.

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Haemostatic Defects in Myelofibrosis

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Abstract. Platelet function, blood coagulation and fibrinolysis were studied in 13 patients with myelofibrosis. The main abnormalities were impaired ADP release and abnormal platelet aggregation with collagen. The delay period was prolonged to a degree varied from a few minutes to indefinitely. Platelet aggregation with ADP was impaired in 3 cases, while adrenaline-induced aggregation was abolished in 7 other patients. Spontaneous changes in the aggregation patterns were also observed. The bleeding time and clot retraction were normal. The platelet factor 3 availability was also normal except in one case. Blood coagulation and fibrinolysis showed variable mild abnormalities. In one case a low-grade chronic disseminated intravascular coagulation was suspected, while a mild hypercoagulable state was present in other cases. The possible role of platelet function and of blood coagulation abnormalities in the interpretation of haemorrhage and thrombosis in myelofibrosis is discussed.

Key Words
Blood coagulation
Fibrinolysis
Myelofibrosis
Platelet function

Bleeding manifestations are not rare in myelofibrosis (MF) [2[illegible]]. They are usually mild, though severe gastrointestinal haemorrhages occasionally occur. Thrombocytopenia accounts for most cases with bleeding; abnormalities of platelet function may also contribute to impaired haemostasis and platelets have been shown to be morphologically abnormal [3, 13, 20] and biochemically defective [13], while the platelet adhesiveness has been found impaired [4]. Abnormal platelet aggregation has been reported in patients with myeloproliferative disorders, including a small number of cases with MF [6, 11, 16, 21]. On the other hand, very little information on defects of blood coagulation and fibrinolysis mechanism in MF is available [9, 1[illegible]]. In general, the haemostatic defects have not been sufficiently defined; the few relevant reports deal

* [illegible] number of cases.

[1] [illegible]

In this work we have studied 13 patients with MF as regards platelet function blood coagulation and fibrinolysis

Material and Methods

Thirteen patients with MF were included in the study The diagnosis was established by conventional clinical and laboratory criteria The salient features were chronic anaemia morphological abnormalities of the red cells (anisopoikilocytosis tear-drop cells etc) leukoerythroblastosis marked splenomegaly hypocellular bone marrow on aspiration and fibrotic marrow on bone biopsy

Two patients (No 10 and 12) had findings of liver cirrhosis Six (No 1-3 6, 9 10) had bleeding manifestations (easy bruising in 4 epistaxis in 3 and gastrointestinal bleeding in 1) and two (No 4 and 9) had thrombotic episodes (splenic infarct and thrombophlebitis of the leg respectively) In 3 out of 13 cases platelet investigation was not completed for various reasons (case 11 was on aspirin throughout his hospitalization case 4 refused further investigation and in case 7 platelet rich plasma free of red blood cells could not be obtained) Also blood coagulation and fibrinolysis tests were omitted in 2 cases because of the presence of liver failure

During the period of investigation ranging from 1 to 10 months no drug was administered to the patients especially aspirin or other drugs affecting platelet function Occasional blood transfusion was given In the latter case the tests were performed at least 1 week later

Platelet Investigation

The bleeding time (BT) was tested by the technique of IVY *et al* [12] and the clot retraction (CR) was measured according to MACFARLANE [14] Platelets were counted as described by BRECHER and CRONKITE [4]

Blood was anticoagulated with trisodium citrate 3.2% the amount of anticoagulant was adjusted to blood haematocrit (PCV) according to the formula

$$x = \frac{100 - PCV}{55}$$

in which x was the anticoagulant volume (ml) added to blood to make a final volume of 10 ml Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared by differential centrifugation [18]

The platelet aggregation and adhesiveness tests and the ADP release determination are described elsewhere [18 19] The degree of aggregation was expressed as a percentage of maximal fall in optical density In the collagen induced aggregation the delay period and the slope of the aggregation curve were also estimated The adrenalin induced aggregation consisted of two phases normally the depth of each phase graded 0-4+ was 1-3+ for the first phase and 2-4+ for the second one

The platelet factor 3 availability (PF 3a) was assessed by the method of WEISS [25]

Blood Coagulation and Fibrinolysis Studies

Venous blood was collected and anticoagulated with trisodium citrate 3.8% as above, for various coagulation and fibrinolysis tests. PPP was obtained by centrifugation at 3000 rpm for 30 min at 4 °C and kept in [illegible] until testing (≤ 2 h).

Prothrombin time (PT), thrombin time (TT), reptilase time (RT) and partial thromboplastin time with kaolin (PTTK) were performed by standard methods [7]. Fibrinogen was measured according to the method of Ratnoff and Menzie [21]. Factors V, VIII and X were assayed according to the methods of Stormorken [22], Hardisty and MacPherson [10] and Denson [[illegible]] respectively. Antithrombin III (at III) activity was assessed after Abildgaard *et al.* [1]. The euglobulin clot lysis time was employed for the study of plasma fibrinolytic activity [24]. The plasminogen was assayed according to Alkjaersig *et al.* [2] and the fibrin degradation products (FDP) after Merskey *et al.* [15].

Results

Platelet Investigation

The results of the platelet count, BT and CR are shown in table I. The platelet count was normal, increased or decreased. In 2 cases (No. 2 and 3) it varied widely during the period of observation. The BT and CR were normal.

Platelet aggregation in all cases studied was impaired (table II). The abnormal aggregation patterns in most cases remained unchanged throughout. In 3 cases (No. 1, 3, 5) spontaneous changes of the aggregation patterns on several occasions were seen.

Collagen-induced aggregation was abnormal except in case 9. The abnormality consisted in absence of aggregation in 3 cases, and occasionally absence of aggregation in 2 other cases. In these cases the platelets observed by phase contrast microscopy did not show any adherence to such collagen fibrils. In the remaining cases the delay period was prolonged (54–150 sec) and the slope of the aggregation curve reduced (32–65%). In the latter cases the degree of aggregation was reduced in all but 2 cases (No. 8 and 9).

The aggregation with ADP was abnormal in cases 2, 12 and 13, occasionally abnormal in cases 1 and 3 and normal in the others. The abnormality consisted in a variable degree of incapacity of platelets to respond to the agent. In case No. 2 a [illegible] of the agent did [illegible] the amplitude of the tracing and the aggregation was not followed by disaggregation.

Adrenaline-induced aggregation was absent in 8 cases, occasionally absent in 2 cases and normal in the others.

In this work we have studied 13 patients with MF as regards platelet function, blood coagulation and fibrinolysis.

Material and Methods

Thirteen patients with MF were included in the study. The diagnosis was established by conventional clinical and laboratory criteria. The salient features were chronic anaemia, morphological abnormalities of the red cells (anisopoikilocytosis, tear-drop cells, etc.), leukoerythroblastosis, marked splenomegaly, hypocellular bone marrow on aspiration and fibrotic marrow on bone biopsy.

Two patients (No. 10 and 12) had findings of liver cirrhosis. Six (No. 1–3, 6, 9, 10) had bleeding manifestations (easy bruising in 4, epistaxis in 3 and gastrointestinal bleeding in 1) and two (No. 4 and 9) had thrombotic episodes (splenic infarct and thrombophlebitis of the leg, respectively). In 3 out of 13 cases platelet investigation was not completed for various reasons (case 11 was on aspirin throughout his hospitalization, case 4 refused further investigation, and in case 7 platelet rich plasma free of red blood cells could not be obtained). Also blood coagulation and fibrinolysis tests were omitted in 2 cases because of the presence of liver failure.

During the period of investigation, ranging from 1 to 10 months, no drug was administered to the patients, especially aspirin or other drugs affecting platelet function. Occasional blood transfusion was given; in the latter case the tests were performed at least 1 week later.

Platelet Investigation

The bleeding time (BT) was tested by the technique of IVY *et al.* [12] and the clot retraction (CR) was measured according to MACFARLANE [14]. Platelets were counted as described by BRECHER and CRONKITE [4].

Blood was anticoagulated with trisodium citrate 3.2%; the amount of anticoagulant was adjusted to blood haematocrit (PCV) according to the formula

$$x = \frac{100 - PCV}{55},$$

in which x was the anticoagulant volume (ml) added to blood to make a final volume of 10 ml. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared by differential centrifugation [18].

The platelet aggregation and adhesiveness tests and the ADP release determination are described elsewhere [18, 19]. The degree of aggregation was expressed as a percentage of maximal fall in optical density. In the collagen induced aggregation the delay period and the slope of the aggregation curve were also estimated. The adrenalin induced aggregation consisted of two phases; normally the depth of each phase, graded 0–4+, was 1–3+ for the first phase and 2–4+ for the second one.

The platelet factor 3 availability (PF 3a) was assessed by the method of WEISS [25].

Table I. Clinical and laboratory data of patients with myelofibrosis

Case No.	Age	Sex	Haemorrhages	Thrombosis	Platelets ×10³/µl	Bleeding time, min	Clot retraction, %	Platelet factor 3 availability, sec
1	77	M	+		160	3.5 4.5, 4.5	62	45.2
2	54	M	+		55-190	5.5 5.5 5.5	–	42.1
3	74	M	+		20-265	2.0 2.5 2.5	45	31.1
4	60	F		thrombophlebitis of leg	190		52	41.2
5	63	M			420	3.5 3.5, 4.5	60	39.0
6	70	F	+		130	2.5 2.5 4.5	63	47.1
7	62	M			220	3.5 3.5 4.0	41	–
8	64	F			540	2.5 3.0 4.5	53	29.2
9	55	M	+	splenic infarct	520	3.5 3.0 4.5	58	37.0
10	50	M	+		230	2.5 3.0 3.5	45	45.5
11	58	F			290	0.5 3.0 3.5	51	–
12	59	F			110			
13	58	M			240	4.0 5.5 4.0	54	60.8
Normals					150-400	1.0-7.0	43-64	34-43

with thrombocytopenia and deterioration of the coagulation status these episodes were considered as evanescent increase of DIC. Increased FDP were found in 3 other cases. In one of these (No. 9) FDP were elevated during the period of a splenic infarct. In the other 2 cases one or more screening tests of coagulation were also prolonged.

Discussion

Our study of platelet function in MF revealed multiple abnormalities. These mainly concerned defective primary platelet aggregation, which could be the result of an abnormality at the platelet receptor site. The adrenalin-induced aggregation was absent in most of our patients. This abnormality differs from that seen in primary or aspirin-induced thrombocytopathia, in which usually the second wave of aggregation is absent, while the first wave remains normal. The defective ADP-induced aggre-

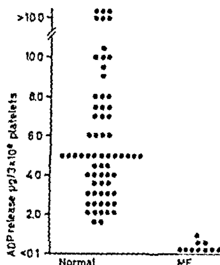


Fig 1 ADP release from platelets by collagen in patients with myelofibrosis (MF) compared to normal

Thrombin induced normal aggregation in all cases tested, except in one, in which on one occasion, the total aggregation was reduced and was followed by disaggregation

ADP release from platelets by collagen was reduced in all cases (fig 1) The PF-3a was normal in all cases except in one (table I)

Coagulation and Fibrinolysis Studies

PT, TT and PTTK were mildly prolonged in 5, 4 and 6 cases, respectively RT was mildly prolonged in 4 cases

Fibrinogen level was consistently low in one case and high in 3 cases Factors V and VIII were low in 2 and 3 cases and high in 1 and 2 cases, respectively Factor X was normal in all cases At-III was below normal levels in 2 cases

Plasma fibrinolytic activity was normal in all cases Plasminogen level was low in one case and FDP were increased in 4 cases

In general, tests of blood coagulation and fibrinolysis were mildly affected However, in one case (No 3) multiple abnormalities were noted namely prolongation of the screening tests of coagulation low levels of fibrinogen, factor V, factor VIII and plasminogen together with increased FDP These findings were consistent with the diagnosis of chronic low-grade disseminated intravascular coagulation (DIC) In fact, this patient from time to time presented generalized bleeding associated

Thus the relationship between abnormal platelet function in *in vitro* testing and bleeding phenomena is not always clear.

Blood coagulation and fibrinolysis studies revealed mild abnormalities in a number of cases. In one patient the findings were consistent with low grade chronic DIC. This complication, confirmed by fibrinogen and platelet kinetic studies, has been reported by BRODSKY *et al.* [5]. In 3 more patients increased amounts of FDP were found, including a patient with splenic infarct which could well be the cause of their increment. Also a hypercoagulable state was found in some patients (high levels of factors I, X, VIII) which could well be the cause of thrombotic episodes which occurred in 2 patients.

The results of these experiments have revealed a number of haemostatic defects in patients with MF. These were platelet dysfunction, thrombocytopenia and abnormalities of blood coagulation, defects which singly may account for the haemorrhagic diathesis observed in most of our patients. The presence of two or more haemostatic defects in the same patient may have a synergistic effect to precipitate bleeding. On the other hand, a hypercoagulable state was found in a number of patients and this may be the cause of thrombotic episodes which occurred in two of them.

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Table II Platelet aggregation in patients with myelofibrosis

	Platelet aggregation by							
	adrenalin		collagen			ADP, %		thrombin
	1st wave	2nd wave	delay per sec	slope degrees	maximum aggregation, %	1 μM	20 μM	%
Case No								
1	0	0	∞	0	0	19	59	–
	0	3+	∞	0	0	2	32	47
	2+	4+	∞	0	0	29	68	82
	0	0	∞	0	0	37	70	88
2	0	0	150	32	53	1	58	–
3	0	0	∞	0	0	8	–	–
	0	0	120	68	61	15	78	81
	0	0	∞	0	0	2	30	70
5	0	0	∞	0	0	16	78	82
	1+	3+	37	75	68	16	78	–
6	1+	2+	120	61	69	25	59	–
8	1+	2+	84	61	66	30	75	–
9	0	0	42	79	80	28	81	–
10	0	0	∞	0	0	12	77	88
12	1+	2+	∞	0	0	1	10	–
13	0	0	108	68	22	0 5	21	–
Normals								
N	25	25	25	25	25	25	25	25
M	–	–	41	78	82	26	75	82
Range	1–3+	2–4+	23–60	70–86	64–100	11–42	58–92	66–98

gation consisted to variable degree of insensitivity of the platelets to the agent, the defect was overcome by increasing the final concentration of the agent Also the reaction between platelets and collagen was *abnormal* in all cases The abnormality consisted mainly in prologation of the delay period indicating defective adherence of platelets to collagen The impaired ADP release from *platelets by collagen, which was a constant* phenomenon in all patients, could also be explained by the defective adherence of platelets to collagen

In all bleeding patients easy *bruising and mucous membrane* haemorrhages were characteristic *of platelet dysfunction* All bleeding subjects had abnormal platelet function tests, but other patients showing the same abnormalities *in vitro* did not present bleeding manifestations

Thus the relationship between abnormal platelet function in *in vitro* testing and bleeding phenomena is not always clear.

Blood coagulation and fibrinolysis studies revealed mild abnormalities in a number of cases. In one patient the findings were consistent with low grade chronic DIC. This complication, confirmed by fibrinogen and platelet kinetic studies, has been reported by Bromsky *et al.* [5]. In 3 more patients increased amounts of FDP were found, including a patient with splenic infarct which could well be the cause of their increment. Also a hypercoagulable state was found in some patients (high levels of factor I, V, VIII) which could well be the cause of thrombotic episodes which occurred in 2 patients.

The results of these experiments have revealed a number of haemostatic defects in patients with MF. These were platelet dysfunction, thrombocytopenia and abnormalities of blood coagulation, defects which singly may account for the haemorrhagic diathesis observed in most of our patients. The presence of two or more haemostatic defects in the same patient may have a synergistic effect to precipitate bleeding. On the other hand, a hypercoagulable state was found in a number of patients and this may be the cause of thrombotic episodes which occurred in two of them.

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Red Cell Life Span in Sickle Cell Trait[1]

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Abstract. Red cell life span was measured in [illegible] subjects with sickle cell trait using both ^{51}Cr and DFP as red cell tags. All were otherwise normal except for one who had pernicious anemia in remission under maintenance therapy. The $T_{1/2}$ ^{51}Cr was 30.3 ± 1.8 days. The mean cell life with DFP was 127.4 ± 12.3 days. Red cell life span in subjects with sickle cell trait living at sea level is normal.

Key Words: Hemoglobinopathies; Red cell survival; Sickle cell trait

While it is commonly accepted that sickle cell trait is not associated with a shortened red cell life span [10], there are scanty and somewhat conflicting data. The earliest studies were done with a modified Ashby technique and the results were normal [4, 13]. Subsequently, 2 subjects studied with the radioactive chromium technique also had normal values [19], whereas the mean $T_{1/2}$ ^{51}Cr of 7 others was distinctly shortened [14]. Although all of the last series were said to have sickle cell trait, the hemoglobin proportions were not recorded and it is possible that at least some had S-thalassemia. Family studies were not reported. More recently, red cell survival was measured in 4 additional subjects with sickle cell trait using both DFP and ^{51}Cr as red cell tags. Essentially normal findings were found [6].

There is also some disagreement about the pathophysiological importance of sickle cell trait. Some authors present clinical observations [illegible, 22]; others [illegible] that most subjects live normal lives with [illegible]

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symptoms [3] Because of increased emphasis on screening programs many more carriers of sickle cell trait are being found, and the answer to this question has become very important [3]

Sickled red cells are rigid and probably mechanically fragile when buffeted about in the circulation. Therefore, if subjects with sickle cell trait have significant sickling *in vivo*, the survival of their red cells should be impaired. Since the available data are somewhat conflicting, this study was planned to determine the red cell life span in subjects with sickle cell trait, comparing the more definitive DF³²P technic with the more commonly used ⁵¹Cr one [7]

Materials and Methods

Five individuals (four females one male) with sickle cell trait participated in the study after giving informed consent. Some were detected in a screening program, one had pernicious anemia in remission and had been treated in the Hematology Clinic with monthly injections of vitamin B_{12} for 8 years, others were found as part of family studies. The age range was 24–69 years. The hematocrit values were within normal limits. Serum iron and percent saturation of transferrin were normal for 4 and slightly reduced but not clearly in the iron deficient range [1] in the other. All subjects but one (pernicious anemia in remission) were normal except for sickle cell trait. The diagnosis of sickle cell trait was based upon a positive test for sickling (metabisulfite or solubility) and the demonstration of more than 50% hemoglobin A and less than 50% hemoglobin S in the hemolysate. For 4 of the 5 subjects the proportions of hemoglobin A and S were determined after electrophoresis and elution from cellulose acetate strips. The proportions of hemoglobin S were 34.8–41.5%. For the other one it was determined by observation of the electrophoretic pattern that there was more hemoglobin A than S. All studies were performed while the patients were in a steady state. The details of the isotopic technics have been described or referred to elsewhere [9]

In addition to determining the best least squares fit for linear and exponential expositions of the data as in the previous study [9] the curves were also fitted to a combined function to detect a combination of random red cell destruction superimposed upon a finite red cell life span [2]. The normal red cell volume for each subject was estimated from body surface area [5]

Results

The red cell mass was within normal limits in each, although in one it was near the lower limit of normal. The T ½ ⁵¹Cr was 30.3 ± 1.8 days

(mean ± standard deviation, individual values 27.3, 30.1, 31.2, 31.4, and 31.5). In our laboratory, 26 days is the lower limit of normal. The mean red cell life with DF³²P was 107.4 ± 12.3 days (95.7, 100.5, 101.1, 114.0, and 125.7). For 3 of the subjects the DF³²P curve fitted the combined function best, for one the linear curve was best (although the combined function was not really different), and for one the exponential curve was best. *In none of them was the difference between the 3 fits statistically significant.* To simplify interpretation (and because for 2 of 3 previously studied patients – see below – the combined curve also fitted best and for the other the difference between any of the fits was inconsequential) we selected the combined curve for each subject. Although one subject was below normal (100–125 days is considered normal) the deviation was not great (95.7 days) and probably not outside of experimental error. The proportion of random red cell destruction was small in each case, 0.09–0.1% per day. The reticulocyte counts were all normal (1.0 ± 0.3%).

Discussion

Our data suggest that there is little or no increased hemolysis in subjects with sickle cell trait who are otherwise normal. This conclusion is in keeping with early studies obtained with the Ashby technic [8, 13] but is somewhat different from those done later with ⁵¹Cr [14, 15]. It is difficult to account for the apparent discrepancy although methodological considerations, diagnostic confusion (e.g., the inclusion of subjects with S-thalassemia among those with sickle cell trait), and the presence of associated diseases might explain it. The site of ⁵¹Cr binding to hemoglobin is the β-chain [11], and it is possible that the abnormal β-chain binds ⁵¹Cr differently than does the normal β-chain. Nevertheless, the rate of ⁵¹Cr elution in patients with abnormal β-chains (S or C) does not seem to be different from that in normal subjects [2]. This also holds true for the patients reported here –

Three of our 5 recently studied subjects and 2 of the 3 previously studied ones [2] had DF³²P red cell survival curves that better fit a combined linear-exponential function (finite life span with superimposed random cell loss) or a linear curve (finite life span). In only one instance [illegible] the previous study did the [illegible] of the [illegible] from the [illegible] line indicate that this better fit was [illegible] the realm of chance. In

symptoms [3] Because of increased emphasis on screening programs many more carriers of sickle cell trait are being found, and the answer to this question has become very important [3]

Sickled red cells are rigid and probably mechanically fragile when buffeted about in the circulation Therefore, if subjects with sickle cell trait have significant sickling *in vivo*, the survival of their red cells should be impaired Since the available data are somewhat conflicting this study was planned to determine the red cell life span in subjects with sickle cell trait, comparing the more definitive DF³²P technic with the more commonly used ⁵¹Cr one [7]

Materials and Methods

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In addition to determining the best least squares fit for linear and exponential expositions of the data as in the previous study [9] the curves were also fitted to a combined function to detect a combination of random red cell destruction superimposed upon a finite red cell life span [2] The normal red cell volume for each subject was estimated from body surface area [5]

Results

The red cell mass was within normal limits in each although in one it was near the lower limit of normal The $T\frac{1}{2}$ ⁵¹Cr was 30 3 ± 1 8 days

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this subject both the finite and the combined curves were significantly better than the exponential one but neither was significantly better than the other. Nevertheless, because the greater number of patients seemed more likely to have a finite red cell survival with a slight amount of random cell loss, we selected that curve for each patient. Hence, the data are interpreted to indicate predominantly finite red cell survival with cell death from old age, accompanied by the destruction of a small proportion of cells at random. Others have found evidence for some random red cell loss in normal individuals but to a greater extent for the female than for the male [2]. We conclude that there does not seem to be sufficient intravascular sickling in subjects with sickle cell trait residing at sea level to produce significantly greater random cell loss than is found in some normal individuals.

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sten wurde die Leukämieentstehung nach Injektion von Virusmaterial aus Chloroleukämien – der typischsten myeloischen Leukämie – gehemmt. Nach den Untersuchungen über die Aufsplitterung [4] bewirken Viruspräparationen aus unreifen myeloischen Leukämien ein breites Spektrum der entstehenden Leukämien. Es ist wahrscheinlich, dass nach Injektion von Virus aus Chloroleukämien der Anteil der eventuell entstehenden lymphatischen, lymphatisch retikulären und Erythroblastenleukämien gehemmt wird, so dass die aufgetretene Hemmung der Chloroleukämie nur scheinbar ist. Das differenzierte Auftreten der Hemmwirkung der Thymektomie bei der Leukämogenese von Myeloleukämien spricht ebenfalls dafür. LEY und GRAFFI [2] und LEY [3] konnten die Bedeutung der Milz für die Genese der myeloischen Leukämie nachweisen. Splenektomie senkte die Anzahl der auftretenden Leukosen auf ein Fünftel des Kontrollwertes. Da der hämatologische Charakter der Leukosen, die noch nach Thymektomie und Virusinjektion von lymphatischem Ausgangsmaterial auftraten (29%), überwiegend myeloisch-retikulär bzw. myeloisch war, sollte versucht werden, Thym- und Splenektomie zu kombinieren, um festzustellen, ob der noch auftretende Anteil der Leukosen im Kombinationsversuch durch Splenektomie gehemmt werden könne. In Ergänzung der Thymektomieversuche wurde versucht, durch Restauration des Thymusmilieus in diesem System die Hemmwirkung der Thymektomie auf die Leukämogenese aufzuheben. Daher wurden thymektomierten und infizierten Tieren Thymuszellen von genetisch gleichwertigen Tieren implantiert.

Material und Methodik

Tiermaterial. Die Mäuse gehörten dem Inzuchtstamm XVII Berlin [illegible] des Stammes XVII vom [illegible] an. Die Leukämieentstehung [illegible]

Thymektomie und Splenektomie. Die Thymektomie wurde an neugeborenen Mäusen im Alter von 1–3 Tagen nach einer modifizierten Methode [illegible] vorgenommen. Die Mortalität nach der Thymektomie [illegible] Alter der Tiere [illegible]

Die Splenektomie wurde nach der Methode von LEY und GRAFFI [illegible] nach der Thymektomie vorgenommen. [illegible]

[illegible]

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Wirkung von kombinierter neonataler Thymektomie und Splenektomie auf die virale Leukämogenese der Maus

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Abstract In previous experiments, murine myeloid leukemia virus has been found to induce only in some cases lymphatic leukemias. The leukemogenesis of the lymphatic types could be strongly inhibited by thymectomy. Leukemias occurring in thymectomized virus infected animals were predominantly of the myeloid type. A combination of thymectomy and splenectomy was used because of the inhibitory effect of splenectomy in virus induced myeloid leukemias. Injection of virus from selected lymphatic leukemias into double-operated animals reduced the incidence of leukemias to 13%. In the bone marrow of intact virus infected double-operated mice, a strong multiplication of the virus has been demonstrated. Using isologous thymic cells in substitution experiments, the inhibitory effect of thymectomy on leukemogenesis could be greatly abolished (80%).

Key Words
Graffi virus
Leukemia viruses
Mouse leukemia
Splenectomy
Thymectomy

In vorhergehenden Untersuchungen wurde die Wirkung der neonatalen Thymektomie auf die Entwicklung von virusinduzierten myeloischen Leukamien [5] und davon abgeleiteten hamatologisch differenten Leukämien untersucht [10]. Aufgrund der Aufsplitterungstendenz der myeloischen Leukamie der Maus [4, 9] konnten als Ausgangsmaterial für derartige vergleichende Thymektomieversuche, ausser den myeloischen Leukamien, auch sehr seltene lymphatische, lymphatisch-retikulare und Erythroblastenleukamien gewonnen werden. Thymektomie bewirkte eine Hemmung der viralen Leukamogenese [10]. Die Grosse der Hemmwirkung hing ab vom hamatologischen Charakter der Ausgangsleukose. Je grosser deren lymphatischer Anteil war, desto starker war die Hemmung. Am wenig

[1] Herrn Dr. BIRKWOLZ und Mitarbeitern danken wir für die Anfertigung der elektronenmikroskopischen Bilder

sten wurde die Leukämieentstehung nach Injektion von Virusmaterial aus Chloroleukämien – der typischsten myeloischen Leukämie – gehemmt. Nach den Untersuchungen über die Aufsplitterung [4] bewirken Viruspräparationen aus unreifen myeloischen Leukämien ein breites Spektrum der entstehenden Leukämien. Es ist wahrscheinlich, dass nach Injektion von Virus aus Chloroleukämien der Anteil der eventuell entstehenden lymphatischen, lymphatisch-retikulären und Erythroblastenleukämien gehemmt wird, so dass die aufgetretene Hemmung der Chloroleukämie nur scheinbar ist. *Das differenzierte Auftreten der Hemmwirkung der Thymektomie* bei der Leukämogenese von Mischleukämien spricht ebenfalls dafür. FEY und GRAFFI [2] und FEY [3] konnten die Bedeutung der Milz für die Genese der myeloischen Leukämie nachweisen. Splenektomie senkte die Anzahl der auftretenden Leukosen auf ein Fünftel des Kontrollwertes. Da der hämatologische Charakter der Leukosen, die noch nach Thymektomie und Virusinjektion von lymphatischem Ausgangsmaterial auftraten (29%), überwiegend myeloisch-retikulär bzw. myeloisch war, sollte versucht werden, Thym- und Splenektomie zu kombinieren, um festzustellen, ob der noch auftretende Anteil der Leukosen im Kombinationsversuch durch Splenektomie gehemmt werden könne. In Ergänzung der Thymektomieversuche wurde versucht, durch Restauration des Thymusmilieus in diesem System die Hemmwirkung der Thymektomie auf die Leukämogenese aufzuheben. Daher wurden thymektomierten virusinfizierten Tieren Thymusstückchen von genetisch gleichwertigen Tieren implantiert.

Material und Methodik

Tiermaterial. Die Mäuse gehörten dem [illegible] XVII [illegible] des Stammes XVII [illegible]

Thymektomie und Splenektomie. [illegible]

Die Splenektomie [illegible]

[illegible]

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Tabelle III. Befundquoten von virusinfizierten thymektomierten Tieren, die Thymuszellen implantiert bekamen und virusinfizierten thymektomierten und Normaltieren

	Auswertbare Tiere	Leukämien	
		Anzahl	%
Thymektomie und Thymuszellen	80	64	80
Thymektomie	30	9	30
Kontrollen	149	140	94

gelang es, die Leukämiequote auf 13% zu senken. Es interessierte uns, ob und in welchem Masse Viren im Gewebe von doppeltoperierten befundfreien Tieren noch zu finden waren. Wir entnahmen daher derartigen alten Tieren Knochenmark zur elektronenmikroskopischen Untersuchung. Wie die Abbildungen 1 und 2 zeigen, wurden in Knochenmarksproben Viruspartikel in grosser Anzahl gefunden. Ebenso konnten in den Zellen häufig Virusabschnürungen beobachtet werden.

Ersatzversuche. Es wurden ebenfalls 3 Versuchsgruppen angesetzt, die das gleiche Virusmaterial aus selektionierten lymphatischen Leukämien injiziert bekamen. In der Versuchsgruppe, in der die Tiere nach der Operation und Virusinjektion Thymuszellen implantiert bekamen, zeigte sich nur eine geringe Wachstumsverzögerung. Als Kontrollen wurden Tiergruppen nur thymektomiert und virusinfiziert sowie Normaltiere mit Virus behandelt (Tab. III).

Diskussion

Die Ergebnisse zeigen zunächst, dass die Kontrollgruppen der Doppeloperationsversuche die erwarteten Befundquoten aufweisen. So entspricht die Leukämiequote, die bei nur thymektomierten virusinfizierten Mäusen [illegible] in etwa den vorangegangenen Versuchen [10]. Mit Hilfe der Doppeloperation gelang es, die Leukämiequote bei virusinfizierten Mäusen auf 13% zu senken. Nachdem die Milz, die als Zielorgan für Viren [illegible] Leukämien fungiert, entfernt worden war, wurde die [illegible] der nach [illegible] Thymektomie [illegible] Leukämien (13% bzw. 3%) [illegible]. Dies [illegible] die Leerbefunde über die Hemmung [illegible] Thymus- und [illegible] Kombinationen [illegible] bestätigt.

maschige Edelstahlnetze gestrichen Die Zellen in der Suspension wurden mit Hilfe der Thomaschen Zahlkammer gezählt Thymektomierten Tieren wurden 24 h nach der Operation und Virusinjektion 1–2mal eine Zellsuspension von 5–8 × 10⁷ Thymuszellen implantiert

Die Versuchsansätze wurden in 3 Gruppen gegliedert Alle Tiere der 3 Gruppen erhielten zum gleichen Zeitpunkt Virus aus lymphatischen Leukämien die aufgrund der Aufsplitterungstendenz der myeloischen Leukämie der Maus [4] erhalten werden konnten In der ersten Gruppe wurden Tiere thymektomiert und splenektomiert Die Verlustquote war sehr hoch Die Tiere entwickelten sich nach den Operationen nur sehr langsam Von der Auswertung ausgenommen werden mussten Tiere mit Thymus bzw Milzresten

Parallel zu dieser Versuchsreihe mit doppeltoperierten Tieren wurden Kontrollversuche mit Tieren angesetzt die nur thymektomiert wurden Zum gleichen Zeitpunkt wurden normale Neugeborene mit Virus injiziert

Ergebnisse

Tabelle I zeigt die Befunde bei doppeltoperierten, nur thymektomierten und Kontrolltieren Tabelle II gibt eine Gegenuberstellung der durchschnittlichen Latenzzeiten der Befunde Mit Hilfe der Doppeloperationen

Tabelle I Befundquoten bei doppeltoperierten, nur thymektomierten und Kontrolltieren nach Injektion von Virus aus selektionierten lymphatischen Leukämien

	Auswertbare Tiere	Leukämien	
		Anzahl	%
Thymektomie und Splenektomie	70	9	13
Thymektomie	39	10	26
Kontrollen	107	101	94

Tabelle II Durchschnittliche Latenzzeiten der Befunde

	Latenzzeiten	
	Tiere ohne Leukämien Tage	Tiere mit Leukämien Tage
Thymektomie und Splenektomie	124	131
Thymektomie	156	119
Kontrollen	–	104

Die Bedeutung des Thymus bzw. der Milz als Targetorgan für eine Leukämieentstehung wird durch die elektronenmikroskopischen Befunde unterstrichen. Es zeigte sich, dass im Knochenmark behandelter und gesplenektomierter, virusinfizierter Tiere eine starke Virusvermehrung vor sich geht. Dennoch kommt es nicht zum Ausbruch einer Leukose, da das entsprechende Erfolgsorgan fehlt.

Aus den Thymusersatzversuchen ist zu ersehen, dass zumindest die annähernde Wiederherstellung des Thymusmilieus durch zelluläre Implantationen die virale Leukämogenese wieder in Gang bringt. Wir erhielten in unseren Versuchen nach Implantation von Thymuszellen neugeborener syngener Spender in thymektomierte, virusinfizierte Tiere eine Leukämierate von 80%. Entsprechende frühere Versuche von Gross [7] und Miller [8] ergaben Befundquoten von 50–100%. Die parallel durchgeführten Kontrollversuche, einmal Injektionen von Virus in normale Neugeborene (94% Befunde) und zum anderen Injektionen in neugeborene thymektomierte Tiere (30% Befunde) im gleichen Versuchsansatz unterstreichen die Tatsache, dass die Hemmung der Leukämogenese durch Thymektomie nach Implantation von Thymuszellen syngener Spender in bestimmtem Masse reversibel ist. Inwiefern die Implantation von Thymusgewebe bzw. Thymuszellen die unterbrochene virale Leukämogenese beeinflusst, ist bis jetzt noch nicht in allen Einzelheiten geklärt. Da eine Implantation von Targetzellen stattgefunden hat, ist anzunehmen, dass dort bevorzugt Transformationsmöglichkeiten bestehen.

Zusammenfassung

In [illegible] Versuchen [illegible] der Maus [illegible] lymphatische Leukämieformen [illegible] Thymektomie [illegible] Knochenmark [illegible]

[illegible] Thymektomie [illegible] Leukämogenese [illegible]



Abb 1 Viruspartikel im Knochenmark befundfreier doppeltoperierter virusinfizierter Tiere (Mause)

Abb 2 Abschnurung eines Viruspartikels im Knochenmark befundfreier doppeltoperierter virusinfizierter Mause Primarvergrosserung 15 000 Sekundarvergrosserung 75 000

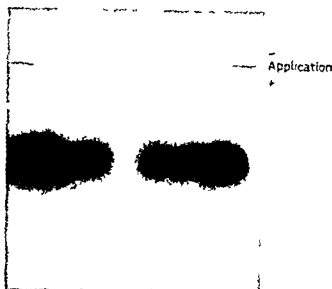


Fig. 1 Paper electrophoresis in Tris EDTA borate buffer pH 8.6 of haemoglobin from the propositus (right) and from a control with a raised Hb A_2 (left)

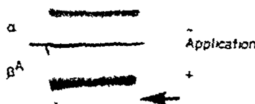


Fig. 2 Starch gel electrophoresis in 6 M urea of the globin from the propositus. The arrow marks the position of the new abnormal β-chain which migrates more rapidly towards the anode than the $β^A$-chain.

component with an abnormal electrophoretic mobility (fig. 1). The level of Hb A_2 was elevated (5.3%), and the alkali-denaturation test [3] revealed the presence of 4.5% Hb F. However, electrophoresis of the globin on starch gel containing 6 M urea [4] showed, in addition to normal α- and β-chains, a new component which migrated more rapidly towards the anode than normal $β^A$-chains (fig. 2). This was presumed to

be an abnormal β-chain more negatively charged than usual. For some reason (see 'Discussion') the additional negative charge appeared to be suppressed in the intact haemoglobin tetramer to such an extent that there was no fast moving component seen on paper electrophoresis. On storage there appeared such a fast moving fraction indicating that there was a change of conformation. Incubation of the haemolysate at 50 °C produced a copious precipitate of unstable haemoglobin [5] amounting to about 10% of the total haemoglobin.

The precipitate was washed with 0.1 M phosphate buffer pH 7.4, redissolved in 0.1 N HCl and converted into globin by precipitation with acid acetone. The globin was then aminoethylated by the method of Jones [6]. Fingerprints of tryptic digests of the aminoethylated (AE) globin were prepared by the technique summarised by Nick et al. [7], and were stained with ninhydrin and specific reagents for histidine, arginine, tyrosine, tryptophan and sulphur-containing amino acids [8].

Figure 3 shows a fingerprint of a tryptic digest of the AE globin with the electrophoretic separation carried out at pH 3.5. A new peptide spot (No. 1), absent from the fingerprint of AE Hb A, was observed located just below the arginine peptide βTp III (No. 2). It was not observed when the electrophoretic separation was carried out at pH 6.4, probably because it was overlapped by other peptides under these conditions. The new spot gave negative reactions with all the specific amino acid reagents. Peptide No. 1 was isolated by preparative fingerprinting [9] at pH 3.5 and further purified by electrophoresis at pH 6.4. Its amino acid composition, shown in table 1, resembled that of βTp XIII (β121–132) except that it lacked tyrosine (β130) and contained one residue of aspartic acid, which is normally absent from βTp XIII. This suggested that the new haemoglobin had a substitution of tyrosine β130 by either aspartic acid or asparagine. However, at pH 6.4 the new peptide migrated towards the anode and was therefore negatively charged, so that the substitution was likely to be Tyr→Asp rather than Tyr→Asn, which would have given an electrically neutral peptide. The normal form of βTp XIII, which gives positive reactions for tyrosine, was also present on the fingerprint (No. 3 in fig. 3) indicating that the precipitate also contained some Hb A as well as the unstable haemoglobin.

Confirmatory evidence for the position of substitution was obtained from the results of carboxypeptidase digestion of the new peptide as follows.

[illegible]

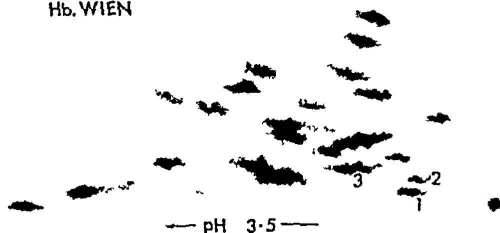


Fig 3 Fingerprint of tryptic digest of the AE globin of a heat precipitate prepared from a haemolysate from the carrier of Hb Wien Origin on right electrophoresis at pH 3 5 1 = Abnormal β-Wien Tp XIII 2 = βTp III, 3 = normal β^A Tp XIII

Table I Amino acid composition of a Wien Tp XIII and carboxypeptidase fragments[1]

Amino acid	1		A1 2	Wn1 2		Wn3	
	A	B		A	B	A	B
Asp	1.2	0	*0.1*	*0.3*		*0.2*	
Thr	0.9	1	1.0	1.1	1	0.9	1
Ser	*0.3*		*0.3*	*0.2*		*0.1*	
Glu	3.0	3	2.1	2.0	2	1.0	1
Pro	2.3	2	1.8	1.8	2	1.8	2
Gly	*0.1*		*0.2*	*0.2*		*0.1*	
Ala	1.8	2	1.5	1.5	1 or 2	*0.1*	
Val	1.0	1	1.0	1.1	1	1.0	1
Tyr	0	1	*0.2*				
Phe	0.9	1	1.0	1.0	1	1.0	1
Lys	0.9	1					

[1] 1 = Abnormal βTp XIII of Hb Wien A = Observed molar ratios for the new peptide B = expected molar ratios for β^A Tp XIII A1 2 Wn1 2 = Peptides β121–129 and 121 128 from carboxypeptidase digests of β^A Tp XIII and β-Wien Tp XIII Wn3 = Peptide β121 126 Each analysis shown in *italics* is considered to represent contamination the values shown in bold type are those which differ in the normal and abnormal forms of βTp XIII

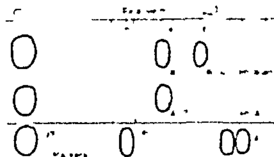


Fig. 4. Electrophoretogram of carboxypeptidase digests of β Wien Tp XIII and β^A Tp XIII. Electrophoresis at pH 6.4, origin at centre. The sequences of the carboxypeptidase fragments are indicated in Figure 5.

of glycine. The supernatant was dissolved in two 0.5 ml aliquots, and each was applied to a 57 × 25 cm sheet of Whatman 3MM paper along a 20 cm line 15 cm from the anodal end of the paper. Electrophoresis in pH 3.5 buffer was then carried out in a [illegible]-cooled [illegible] tank at a voltage of 2.5 kV (potential gradient about 50 V/cm) for 90 min. After drying the electrophoretograms, the area containing β^A Tp III and the new peptide, which had the same electrophoretic mobility at pH 3.5, was located by staining guide strips with ninhydrin and the Sakaguchi reagent. The areas were cut out, sewn on to new sheets of 3MM paper 25 cm from the anodal end, and electrophoresis in pH 6.4 buffer was carried out at 2.5 kV for 60 min to separate β^A Tp III and the abnormal β Tp XIII. The peptide was eluted with 0.5% formic acid and, after evaporation to dryness, the residue was redissolved in water and digested with carboxypeptidases A and B. A similar digest of normal β^A Tp XIII and an enzyme blank were also prepared. The digest was fractionated by paper electrophoresis at pH 6.4 (Fig. 4).

After staining with dilute ninhydrin (0.05% in acetone), both peptide digests showed a spot with the same mobility as free lysine, and a second spot (denoted A1.2 and Wn1.2 in Fig. 4) with an anodal mobility relative to aspartic acid (R_{Asp}) of 0.25. The latter appeared to consist of 2 overlapping spots as indicated by the broken lines in the figure. Amino acid analysis of both spots A1.2 and Wn1.2 (Table 1) gave results indicating that they contained a mixture of peptides corresponding to β121–126 and β121–124 (Fig. 5). After more prolonged digestion with carboxypeptidases A and B, an anodal peptide (R_{Asp} = 0.53) was obtained which had an amino acid composition corresponding to β121–125. [illegible] carboxypeptidase A [illegible] Tp XIII [illegible] digestion [illegible] by the presence of [illegible] position 125.

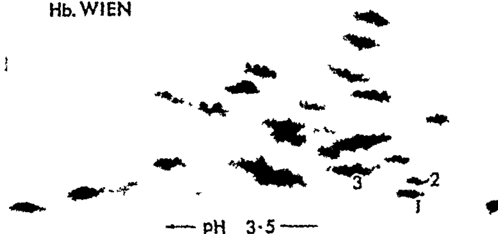


Fig. 3 Fingerprint of tryptic digest of the AE globin of a heat precipitate prepared from a haemolysate from the carrier of Hb Wien. Origin on right, electrophoresis at pH 3.5. 1 = Abnormal β-Wien Tp XIII, 2 = βTp III, 3 = normal β^A Tp XIII

Table I Amino acid composition of a Wien Tp XIII and carboxypeptidase fragments[1]

Amino acid	I		A1, 2	Wn1, 2		Wn3	
	A	B		A	B	A	B
Asp	**1.2**	**0**	*0.1*	*0.3*		*0.2*	
Thr	0.9	1	1.0	1.1	1	0.9	1
Ser	*0.3*		*0.3*	*0.2*		*0.1*	
Glu	3.0	3	2.1	2.0	2	1.0	1
Pro	2.3	2	1.8	1.8	2	1.8	2
Gly	*0.1*		*0.2*	*0.2*		*0.1*	
Ala	1.8	2	1.5	1.5	1 or 2	*0.1*	
Val	1.0	1	1.0	1.1	1	1.0	1
Tyr	**0**	**1**	*0.2*			–	
Phe	0.9	1	1.0	1.0	1	1.0	1
Lys	0.9	1				–	

[1] I = Abnormal βTp XIII of Hb Wien. A = Observed molar ratios for the new peptide. B = expected molar ratios for β^A Tp XIII. A1, 2, Wn1, 2 = Peptides β121–129 and 121–128 from carboxypeptidase digests of β^A Tp XIII and β-Wien Tp XIII. Wn3 = Peptide β121–126. Each analysis shown in *italics* is considered to represent contamination, the values shown in bold type are those which differ in the normal and abnormal forms of βTp XIII

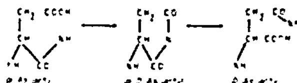


Fig. 6. The α-β rearrangement of an aspartyl peptide via a cyclic intermediate as postulated by Swallow and Abraham [12]

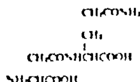


Fig. 7. β-Aspartyl glutamine

is that the peptide bond between Asp 130 and Gln 131 may have undergone an α-β rearrangement via a cyclic intermediate as illustrated in figure 6. The rearrangement would liberate a free α-COOH group at position 130 which might then be susceptible to the action of carboxypeptidase A, possibly more slowly than normal. The products of carboxypeptidase A digestion would be β-aspartyl glutaminyl lysine, free amino acids, Ala and Gln, and a residual peptide (121-126). The action of carboxypeptidase B on β-aspartyl glutaminyl lysine would liberate Lys and a dipeptide, aspartyl glutamine (fig. 7) [illegible] further degradation.

Such α-β [illegible] have been observed [illegible], [illegible] particularly by Swallow and Abraham [12] with a partial peptide from the [illegible] of [illegible] by [illegible] *et al.* [13] and [illegible] by [illegible] [14]. However, the conditions required to bring about the α-β rearrangement are [illegible] e.g. prolonged exposure to strong [illegible] [13] or prolonged boiling of aqueous solution [14]. The haemoglobin was subjected only to brief exposure to 0.1 N HCl prior to precipitation of the globin and to brief heating during precipitation of the core. The tryptic digest was exposed [illegible] during the [illegible]

retic separation at pH 3 5, and elution with 0 5% formic acid (pH about 2 5) Even when 0 5 M NH_4OH was used instead of 0 5% formic acid for elution of β-Wien Tp XIII, *the peptide Wn4 was still obtained in the* carboxypeptidase digest

Discussion

Position β130 (H8) is located at an internal site in the haemoglobin molecule at the base of the haem pocket [27] In human Hb A it is occupied by a tyrosine residue of which the phenyl ring is internal but not in direct contact with the haem group, and the phenolic hydroxyl group emerges in a surface crevice near helix A probably forming a hydrogen bond with the carbonyl group of Val A8(11)β [16] Position H8 is one of approximately 20 positions in the haemoglobin and myoglobin chains which are internal and occupied by non polar residues which are not in direct contact with the haem groups [15] The other positions in the helical notation are A8, 11, 12, 15, B6, 9, 10, 14, D5, F4, 8, 12, 18, 19, F1, G11, 12, 16, H11, 12 and 15, except that in the α-chain D5 is absent, and H12 is a haem contact, and in the β chain H15 is a haem contact Except for position B6, which is an almost invariant Gly, these positions show a greater degree of inter species variation than do the *residues in direct contact with the haem* but with a few exceptions they are occupied by non polar side chains Position H8 is occupied by Leu in the α-chains, by Tyr or Phe in the β-chains of some mammalian haemoglobins, *by Trp in the γ-chain of human Hb F, and by Met in all known* mammalian myoglobins Table II lists the known haemoglobin variants with substitutions at these internal non polar sites These all show signs of instability varying from the slight instability *of Hb Sogn [17]* which is not associated with any clinical signs of haemolytic anaemia, to the more severe instability associated with the typical symptoms of unstable haemoglobin haemolytic anaemia In Hb Sogn β14 A11 Leu-Arg the guanidinium group of the arginine may be accommodated in a surface crevice without causing great disruption of the structure [16] Presumably, this is not so readily accomplished in Hb Ann Arbor α80 F1 Leu Arg which is associated with a definite haemolytic anaemia [26] In Hb Genova β28 B10 Leu Pro [20] and I Perth β32 B14 Leu Pro [22] the substitutions do not introduce side chain into the interior, but cause disruption of the B I verdale-Bronx β h-

Table II. Abnormal haemoglobins with substitutions at internal nonpolar sites

				Reference
β14	A11	Leu→Arg	Hb Sogn	[17]
β24	B6	Gly→Arg	Hb Riverdale-Bronx	[18]
β24	B6	Gly→Val	Hb Savannah	[19]
β28	B10	Leu→Pro	Hb Genova	[20]
β28	B10	Leu→Gln	Hb St Louis	[21]
β32	B14	Leu→Pro	Hb Perth†	[22, 23]
β74	E18	Gly→Asp	Hb Shepherds Bush	[24]
β85	F1	Phe→Ser	Hb Buenos Aires	[25]
α80	F1	Leu→Arg	Hb Ann Arbor	[26]
β130	H8	Tyr→Asp	Hb Wien	

† A second finding of an identical haemoglobin called Hb Abraham Lincoln has been reported by Honig *et al* [23].

Arg [18] and Hb Savannah β24 B6 Gly→Val [19] the introduction of bulky side chains of either polar or non-polar nature into a closely packed region of the molecule between the B and E helices causes disruption of the structure by forcing these helices apart. In Hb St Louis β28 B10 Leu→Gln [21], Hb Shepherds Bush β74 E18 Gly→Asp [24], Hb Ann Arbor and Hb Buenos Aires β85 F1 Phe→Ser [25], polar groups are introduced into the interior causing instability of the haemoglobin with associated haemolytic anaemia.

Although the Tyr→Asp substitution in Hb Wien would be expected to increase the negative charge of the molecule, it does not easily separate from Hb A on electrophoresis at pH 8.6. Pietsch and Lehmann [17] have suggested that this might be accounted for by the formation of a new internal positive charge by a rise in pK of a previously uncharged group. This might be either the imidazole group of His β(NA2) or the amino group of Val β(NA1). The movement of either of these or of the [illegible] of Asp β130 would be likely to cause extensive conformational changes. Six residues of the β H helix, H1, 2, 3, 5, 6 and 9, form part of the α₁β₁ interface [27] and make several atomic contacts with atoms in the side chains of residues B11, 12, 15 and 16 of the α chain. Any such distortion of the H helix would be expected to greatly weaken the α₁β₁ contacts and favour dissociation into monomers, which are [illegible] [illegible] [illegible].

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Normal Factor VIII Antigen Level in Combined Congenital Deficiency of Factor V and Factor VIII

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Key Words
Blood coagulation
Factor VIII antigen
Factor V deficiency
Factor VIII deficiency

As a result of immunological studies several conditions due to structural abnormalities of clotting factors have been described. Only one patient with congenital combined factor V and factor VIII deficiency has been investigated immunologically so far and found to have a normal factor VIII antigen level [1].

We have recently studied another patient with this peculiar disease [2] and were therefore able to carry out some immunological studies on whole plasma [3]. The factor VIII activity was 14% of normal, yet immunologically the factor VIII level was >100% (fig. 1). An antiserum obtained from rabbits immunized with human factor VIII was used (Behringwerke).

Therefore, it seems that this peculiar disease is associated with normal or even elevated factor VIII antigen levels and low factor VIII activity. The discrepancy with biological factor VIII activity seems similar to that found in classical hemophilia A [1].

Some genetic considerations seem justified, namely that both sex-linked and autosomal recessive factor VIII deficiences are associated with low factor VIII activity and normal or elevated factor VIII antigen. On the contrary, autosomal dominant conditions (von Willebrand's disease)

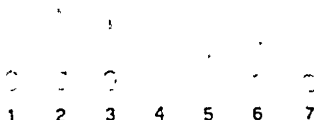


Fig. 1. Electroimmunoassay (Laurell) in (1) undiluted von Willebrand's disease plasma, (2) undiluted hemophilia A plasma, (3) undiluted combined factor V and factor VIII deficiency plasma, (4)-(7) 1:8, 1:4, 1:2, and undiluted pooled normal plasma, respectively.

are associated with low factor VIII biological activity and low factor VIII antigen.

References

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3 Girolami, A.; Sticchi, A.; Barbui, T., and Barbieri, G.: Factor VIII immunological assay. An evaluation of several methods using whole plasma. Blut (in press).

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Normal Factor VIII Antigen Level in Combined Congenital Deficiency of Factor V and Factor VIII

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Key Words
Blood coagulation
Factor VIII antigen
Factor V deficiency
Factor VIII deficiency

As a result of immunological studies, several conditions due to structural abnormalities of clotting factors have been described. Only one patient with congenital combined factor V and factor VIII deficiency has been investigated immunologically so far and found to have a normal factor VIII antigen level [1].

We have recently studied another patient with this peculiar disease [2] and were therefore able to carry out some immunological studies on whole plasma [3]. The factor VIII activity was 14% of normal, yet immunologically the factor VIII level was >100% (fig. 1). An antiserum obtained from rabbits immunized with human factor VIII was used (Behringwerke).

Therefore, it seems that this peculiar disease is associated with normal or even elevated factor VIII antigen levels and low factor VIII activity. The discrepancy with biological factor VIII activity seems similar to that found in classical hemophilia A [1].

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1 2 3 4 5 6 7

Fig. 1. Electroimmunoassay (Laurell) in (1) undiluted von Willebrand's disease plasma; (2) undiluted hemophilia A plasma; (3) undiluted combined factor V and factor VIII deficiency plasma; (4)–(7) 1:8, 1:4, 1:2, and undiluted pooled normal plasma, respectively.

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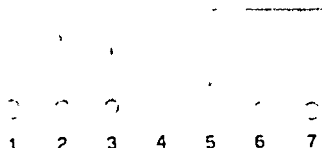


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